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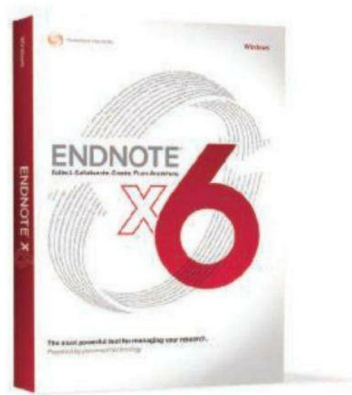
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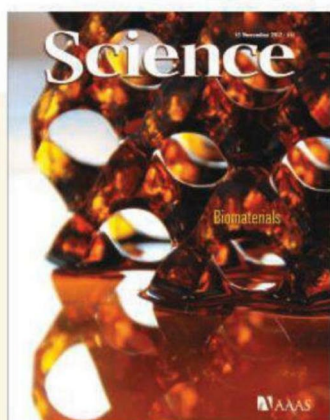
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A 36× enlarged triply periodic porous cube of photocured polymer, 60 millimeters in total length, shown reflected off a pool of uncured resin. Computer-aided design makes it possible to tailor materials with control over porosity, pore size, and mechanical properties. These materials may subsequently find use as scaffolds for tissue engineering and cell-laden hydrogel constructs. See the special section starting on page 899 for a series of articles on biomaterials.

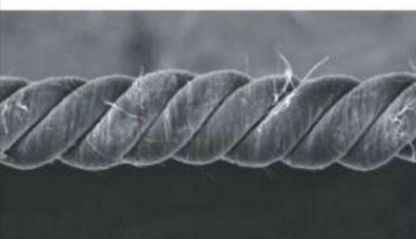
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Publication Ahead of Print

C/EBP Transcription Factors Mediate Epicardial Activation During Heart Development and Injury

G. N. Huang et al.

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B. J. O'Roak et al.

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The COMPASS Subunit Spp1 Links Histone Methylation to Initiation of Meiotic Recombination

L. Acquaviva et al.

10.1126/science.1225739

Porphyry-Copper Ore Shells Form at Stable Pressure-Temperature Fronts Within Dynamic Fluid Plumes

P. Weis et al.

10.1126/science.1225009

Optomechanical Dark Mode

C. Dong et al.

10.1126/science.1228370

Alignment of Magnetized Accretion Disks and Relativistic Jets with Spinning Black Holes

J. C. McKinney et al.

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Two single amino acid changes enable the adaptor protein TRAF5 to promote antiviral responses.

RESEARCH ARTICLE: The Tetraspanin CD37 Orchestrates the $\alpha\beta 1$ Integrin-Akt Signaling Axis and Supports Long-Lived Plasma Cell Survival

A. B. van Spriel et al.

Antibody-producing B cells require CD37-dependent integrin signaling for long-term survival.

PERSPECTIVE: PTEN—An Intercellular Peacekeeper?

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Transfer of PTEN between cells has potential as an intercellular form of tumor suppression.

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COMMENTARY: What Is the Greatest Regulatory Challenge in the Translation of Biomaterials to the Clinic?

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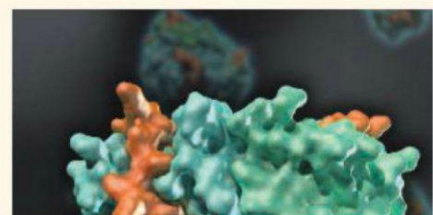
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Ancient Weaponry

Hafting, which allowed projectile points to be attached to a staff, was an important technological advance that greatly increased the functionality of weapons of early humans. This technology was used by both Neandertals and early *Homo sapiens* and is readily seen after about 200,000 to 300,000 years ago, but whether it was used by a common ancestor or was separately acquired by each species is unclear. Supporting use by a common ancestor, Wilkins *et al.* (p. 942) report that stone points in a site in central South Africa were hafted to form spears around 500,000 years ago. The evidence includes damaged edges consistent with this use and marks at the base that are suggestive of hafting.

Nanotube Yarn Actuators

Actuators are used to convert heat, light, or electricity into a twisting or tensile motion, and are often described as artificial muscles. Most materials that show actuation either provide larger forces with small-amplitude motions, such as the alloy NiTi, or provide larger motions with much less force, such as polymeric materials. Other problems with such actuators can include slow response times and short lifetimes. Lima *et al.*

(p. 928, see the Perspective by Schulz) show that a range of guest-filled, twist-spun carbon nanotube yarns can be used for linear or torsional actuation, can solve the problems of speed and lifetime, and do not require electrolytes for operation.

Channels from DNA

Artificial transmembrane channels are of interest for applications, such as sensing and modifying cell signaling. Langecker *et al.* (p. 932; see the Perspective by Strano) used α -hemolysin as a model for creating a nanostructure with DNA origami that, when inserted into a lipid bilayer membrane, acted as a membrane channel. Ion channel responses were similar to those measured for natural ion channels, and channels that protruded further into the membrane exhibited greater gating responses. The channels were used to detect single-DNA molecules.

Coherent Heat Flow

Typically, heat in solids is transported incoherently because phonons scatter at interfaces and defects. Luckyanova *et al.* (p. 936) grew superlattice films made from one to nine repeats of layers of GaAs and AlAs, each 12-nm thick. Thermal conductivity through this sandwich structure increased linearly with the number of superlattice repeats, which is consistent with theoretical simulations of coherent heat transport.

Magnetic Pallasites

The origin of pallasite meteorites seems to defy explanation because their main constituents—iron and olivine—should have segregated into layers inside their parent body. The generally accepted model suggests that they formed at the core-mantle boundary of an asteroid. Tarduno *et al.* (p. 939; see the Perspective by Weiss) measured a remnant magnetization in olivine crystals of two pallasite meteorites and conclude that a dynamo must have operated in their parent body, providing further evidence that some asteroids were capable of dynamo generation. The data, together with thermal modeling, suggest that some pallasites could have formed when liquid FeNi from the core of an impacting asteroid was injected into the mantle of a large protoplanet.



Experience Versus Models

There is an ongoing debate over what the orbitofrontal cortex contributes to behavior, learning, and decision-making. Jones *et al.* (p. 953) found that the orbitofrontal cortex was important for value-based computations when value must be inferred from an associative model of the task but not when value estimates based on previous experience are sufficient. This result calls into question the assumption that this region simply signals economic value. However, it would be consistent with a concept of the orbitofrontal cortex as being important for constructing model-based representations of the world that are orthogonal to value.

Getting Autophagy to Akt

The protein kinase Akt is often activated in human cancers and is thought to promote tumor formation. One way in which it may do so is to inhibit autophagy (a process by which the cell digests its own proteins or organelles, especially damaged ones). Wang *et al.* (p. 956, published online 25 October; see the Perspective by Koren and Kimchi) provide a direct molecular mechanism by which Akt regulates autophagy. Beclin, a component of the autophagy machinery, appears to be a direct target of phosphorylation by Akt. Such phosphorylation enhanced interaction of Beclin with intermediate filaments of the cytoskeleton and inhibited autophagy. Expression of a modified Beclin 1 molecule that could not be phosphorylated by Akt inhibited Akt-induced transformation of cells in culture and tumor formation in a mouse model.

Removing Typhoid Restriction

Some bacterial pathogens exhibit exquisite host adaptation and can only infect a single host. For example, *Salmonella enterica* serovar Typhi (*S. Typhi*), the cause of typhoid fever, can only infect humans. The host restriction is manifested at the cellular level because *S. Typhi* is unable to survive within macrophages of species other than human. Spanò and Galán (p. 960) found that expression of a single type-III secretion effector protein from a broad host *Salmonella* in *S. Typhi*, allowed this human-exclusive pathogen to survive within macrophages from a nonpermissive host. Furthermore, *S. Typhi* expressing this effector was able to replicate within mice, a nonpermissive host.

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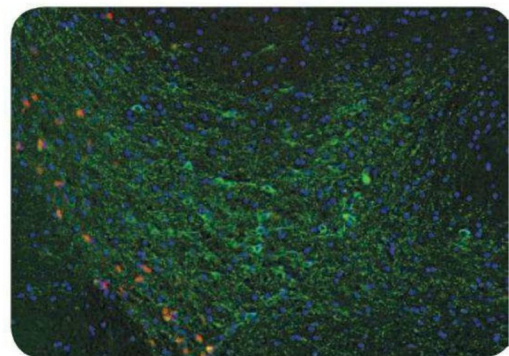
How to Hear

In mammalian ears, a chain of biophysical events allows for the translation of airborne acoustic energy into mechanical vibrations that can be detected by mechanosensory cells in the cochlear. Mammalian ears have been considered largely unique in this transformation because it is the delicate, mammalian-specific, bones of the middle ear that facilitate this transmission, known as impedance conversion. In most other vertebrates the bones that make up the middle ear function as part of the lower jaw, limiting the potential for vertebrate convergence in this process. However, **Montealegre-Z. et al.** (p. 968, see the Perspective by **Hoy**) show that the rainforest katydid, an insect with ears on its hind legs, have converged on a similar three step impedance conversion process using a unique tympanum-lever organ. This convergence, across millions of years of evolutionary distance, shows the flexibility of morphology to adapt to biophysical challenges.

Synthetic Parkinson's

Parkinson's disease (PD) and related α -synucleinopathies are defined by the accumulation of α -synuclein (α -Syn)—containing intraneuronal inclusions—Lewy bodies (LBs) and Lewy neurites (LNs)—in association with the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and other brain regions. However, a cause-and-effect relationship between LB/LN formation and neurodegeneration remains unclear. Indeed, whether LB/LNs are toxic or represent a neuroprotective response has been contentious. **Luk et al.** (p. 949)

injected α -Syn fibrils generated from recombinant mouse α -Syn protein into the dorsal striatum of wild-type mice and found that misfolded α -Syn caused the formation of PD-like LB/LNs and subsequent cell-to-cell transmission of pathologic α -Syn to anatomically interconnected regions, including the SNpc. Furthermore, the formation of LB/LNs and their accumulation in SNpc resulted in the progressive loss of these dopaminergic neurons, reduced dopamine innervations to the dorsal striatum, and culminated in motor deficits similar to PD. Thus, a synthetic misfolded wild-type protein (that is, α -Syn) was able to elicit and transmit disease pathology and neurodegeneration in healthy nontransgenic mice.



Artificially Induced Oocytes

In mice, male embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been shown to differentiate into primordial germ cell-like cells (PGCLCs) in vitro. Upon transplantation into testes, these PGCLCs can form fully functional sperm. Again working in mice, **Hayashi et al.** (p. 971, published online 4 October) found that female ESCs and iPSCs can also differentiate into PGCLCs, which, when aggregated in reconstituted ovaries, exhibited epigenetic reprogramming and meiotic potential in vitro. Upon transplantation of the reconstituted ovaries under ovarian bursa, female PGCLCs developed into fully grown oocytes that contributed to healthy offspring upon in vitro maturation and fertilization.

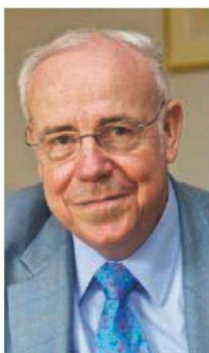
Helping T Helper Transcription

Members of the interferon response family of transcription factors (IRFs) are specifically expressed in immune cells and are known to regulate their differentiation. IRF4 and IRF8 regulate gene expression by binding to other transcription factors, which results in their recruitment to composite motifs in the genome. Although the specific mechanism of how this regulation works in some immune cells is understood, how it occurs in T cells is not clear because the transcription factors that normally partner with IRFs are absent. Using genomic analysis, **Glasmacher et al.** (p. 975, published online 13 September; see the Perspective by **Martinez and Rao**) now identify IRF4-AP-1 composite elements in T helper 17 (T_H17) cells and show that IRF4 and the AP-1 factor Batf cooperatively assemble on a large array of genes required for T_H17 cell differentiation and function. Assembly of such heterodimers was also observed in T_H2 cells, B cells, and dendritic cells, which suggests the general importance of this motif in immune cell differentiation.

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Indira Nath is Raja Ramanna Fellow and Emeritus Professor at the National Institute of Pathology, Indian Council of Medical Research, New Delhi, India. E-mail: indiranath@gmail.com.



Ernst-Ludwig Winnacker is Secretary General of the Human Frontier Science Program, Strasbourg, France. E-mail: elwinnacker@hfsp.org.

Responsible Research Conduct

LAST MONTH, THE INTERACADEMY COUNCIL (IAC) AND THE INTERACADEMY PANEL (IAP), THE GLOBAL network of science academies, released a report on responsible behavior in science.* The recommendations of the committee, which we chaired, were inspired by several trends in today's research environment that have made it an exciting time to do research, but have also made science a more complicated enterprise, raising concerns about ethical conduct and values.

By working collaboratively, researchers can hope to answer questions never addressed before, including those with substantial influence on society. At the same time, today's international, interdisciplinary, team-oriented, and technology-intensive research has created an environment more fraught with the potential for error and distortion. Technology makes it easier to plagiarize or manipulate data in misleading ways. Researchers collaborating on interdisciplinary problems in different countries may have varied ethical perceptions due to differences in disciplines as well as legal traditions and cultures. The increasing pressure on scientists to publish in high-impact journals to drive favorable hiring and promotion decisions also creates incentives for misconduct.

Establishing codes of conduct in science is not new: National and international organizations have issued a range of guidelines to ensure responsible conduct in research.† The goal of the IAC-IAP committee was to establish principles that transcend disciplinary or national boundaries. The project began with seven fundamental values that apply universally in scientific research as well as in daily life: honesty, fairness, objectivity, reliability, skepticism, accountability, and openness. Based on these values, our committee specified principles that apply at different stages of the research process, from the development of the research plan to the reporting of results. The responsibilities not only of scientists but also of institutions in the research enterprise are described in detail. For example, research organizations must establish mechanisms for addressing allegations of irresponsible behavior and for protecting whistleblowers, with ombudsmen or designated agencies that researchers can consult when concerns or questions arise. Scientific journals must make retractions highly visible to minimize the continuing citation of retracted material. And we strongly suggest the establishment of independent oversight bodies to guard against face-saving cover-ups by institutions.

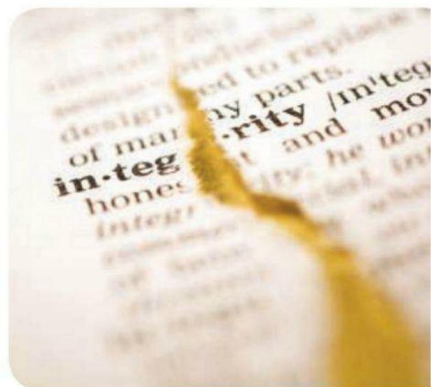
Our committee also emphasizes the role of national academies as forceful leaders on all issues involving responsible conduct in research. They are not only prestigious but also independent organizations involved in research issues. Thus, they have the responsibility to establish and disseminate the necessary standards of ethical scientific conduct. The direct involvement of national academies should bolster the efforts of research organizations to remain rigorous in enforcing standards, while counteracting the temptation of institutions to downplay instances of misconduct. The report also examines the contentious distinction between research and advocacy, recently highlighted by the publication of controversial research concerning the potential dangers of genetically modified corn.‡ Researchers have the right to express their opinions and seek to influence public policy. But they need to be careful to distinguish between their roles as specialists and as advocates. Those who choose to be advocates have a responsibility to themselves and to the research community to be transparent about the financial support they receive for the statements they make, as well as about any conflict of interests.

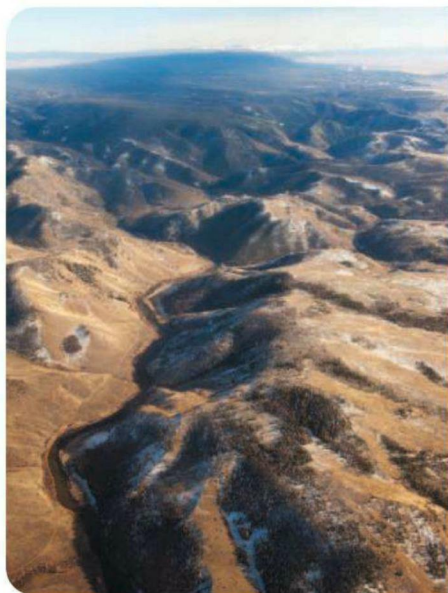
The IAC-IAP report and similar efforts, such as the statement released in 2010 by the 2nd World Conference on Research Integrity,§ are just the initial steps toward a truly global set of standards for the research community. They not only chart areas of widespread agreement but also point to the important issues that remain to be resolved to establish consistent expectations for researchers worldwide.

— Indira Nath and Ernst-Ludwig Winnacker

10.1126/science.1231306

*www.interacademies.net/10878/19787.aspx. †www.allea.org/Pages/ALL/24/581.bGFuZz1FTkc.html. ‡G.-E. Seralini *et al.*, *Food Chem. Tox.* **50**, 4221 (2012); <http://scim.ag/FranceGM>. §www.singaporestatement.org.





ECOLOGY

Rivers of Gene Flow

Antibiotic resistance is becoming an urgent problem. We point one finger at lax medication sales, and another at livestock husbandry, but there is also an ancient background signal of antibiotic resistance, in part stemming from the natural functions of these molecules in the soil. Even if we control the clinical use of antibiotics, there is still a growing problem of resistance. Where is it coming from? Pruden *et al.* have made the enormous undertaking of identifying environmental sources of antibiotic resistance genes in one river system from pristine sources in the Rocky Mountains, flowing through not just an altitudinal gradient but also a gradient of human activity. The background signal was measured by the occurrence of the *tet(W)* (tetracycline resistance) gene, whereas the *sul1* (sulphonamide resistance) gene was taken as a marker of anthropogenic contamination. The results unambiguously showed accumulation of the *sul1* gene down the river basin, primarily contributed by upstream animal feeding operations, with a modest input from wastewater treatment. — CA

Environ. Sci. Technol. **46**, 11541 (2012).

PHYSICS

Spin-Cavity Computing

Today's (very rudimentary) quantum computers come in different guises, each with their own set of pros and cons. A growing trend has been to put two different technologies together in hybrid architectures in order to have the best of both worlds. One of the aims of such initiatives is to realize long-distance coupling between spin qubits (quantum bits based on electron spins) in quantum dots (zero-dimensional semiconductor nanostructures that allow controlled coupling of one or more electrons) via interactions with a superconducting microwave cavity. Petersson *et al.* made progress toward that goal by coupling a double quantum dot in an InSb nanowire to the electric field of a cavity, taking advantage of the strong spin-orbit interaction of InSb. The coupling was demonstrated by using a pulse sequence to electrically control the spin state, which was then read out in the phase response of the cavity. The estimated spin-cavity coupling is still shy of the strong limit required for two distant spin qubits to communicate; however, it is expected that improving the quality of the cavity and the coherence of the qubit, and/or using a material with stronger spin-orbit interactions, will bring physicists closer to that goal. — JS

Nature **490**, 380 (2012).

CLIMATE SCIENCE

The Shape We're In

Climate is warming—there is no doubt about it—but how fast is it happening? Different regions are warming at different rates, but the one where temperatures are increasing most

rapidly is the Arctic, making it an important region to understand. Based on data from three different satellite sensors, together with temperature measurements from a number of weather stations and field observations of snow and ice conditions, Nghiem *et al.* report that surface melting extended across more than 98% of the Greenland Ice Sheet in July 2012. This is a rare event (similar episodes are known to have occurred only twice in the past 800 years) and, in isolation, it cannot be understood as part of a trend. However,



because there is so much frozen water in the Greenland Ice Sheet and sea-level rise from its melting is a potentially serious problem, it will be important to determine how often, how extensively, and why such extreme melt events occur. Being able to observe these events so soon after they occur could provide additional opportunities to determine their impacts more explicitly and thus to reveal more clearly the shape we're in. — HJS

Geophys. Res. Lett. **39**, L20502 (2012).

CLIMATE SCIENCE

Less Snow in the Arctic

At high northern latitudes, the amount and timing of snow melt in spring have important consequences; for example, for water availability, climate, and ecosystem processes. However, snow cover in these regions varies widely in time and space, complicating monitoring efforts. Derksen and Brown used a weekly snow chart time series produced by the U.S. National Oceanic and Atmospheric Administration since 1967, mostly from satellite data, to analyze trends in snow cover in the Arctic during spring. For May and June, the record shows statistically significant reductions in snow cover for both North America and Eurasia. Snow cover loss accelerated since around 2000, with record lows set in the past 5 years. Overall, snow cover over Northern Hemisphere land areas in June between 1979 and 2011 has been lost at a rate of 17.8% per decade. These losses exceed those projected by state-of-the-art models and are also larger than sea ice losses (10.6% per decade since 1979). The results contribute to the emerging evidence for an accelerated response at high latitudes to global warming. — JFU

Geophys. Res. Lett. **39**, L19504 (2012).

ECONOMICS

Teaching Texting

Mobile phones are abundant in developing countries; why not use them to teach reading and math in place of the far more cumbersome and fragile channels of books and computers? Aker *et al.* describe the results of a randomized

experiment in Niger, one of the poorest countries in the world, with one of the lowest rates of adult literacy and numeracy. In half of the 113 villages studied, adult students participating in the same education program received nonsmart mobile phones, with one phone per group of five students and instruction in sending and receiving short messages. Tests before and after the program showed that the students improved from a pre-class beginner level (no literacy or numeracy) to grade one in writing and grade two in math, with those in the mobile phone classes scoring 10% higher. Although proficiency declined somewhat during the 7-month agricultural season, the differential between the phone and phoneless students persisted. Furthermore, the combination of teaching and mobile phone access also resulted in more calling and messaging (both of which rely on numbers, symbols, and letters). Because initiating calls is expensive in Niger, whereas receiving calls is free, providing students with knowledge and means may help to solidify classroom gains. — GJC

Am. Econ. J. Appl. **4**, 94 (2012).

GEOLOGY

Tsunamis of the Past

According to some seismic hazard maps, a large earthquake was not expected to occur offshore of the Tohoku region in Japan. The occurrence of the 2011 M_w 9.0 Tohoku-oki earthquake—one of the largest earthquakes in

occurrence of another large earthquake in 869 CE, often called the Jogan earthquake. Sawai *et al.* synthesized sedimentary data, including diatom assemblages and radiocarbon dates, of nearly 400 new and previously identified paleotsunami deposits from the Jogan and other historical earthquakes along the coast of Japan. By combining observations of coseismic subsidence in the sediment with tsunami simulation models, the authors estimate that the size of the Jogan earthquake was at least M_w 8.4, and the resultant tsunami reached at least 1.5 km inland. With a recurrence interval of 500 to 800 years, large earthquakes in this region of Japan do not occur frequently but are more likely to occur than predicted by previous hazard models. — NW

Geophys. Res. Lett. **10.1029/2012GL053692** (2012).

GENETICS

Genome Packaging with a Bow

The genomes of prokaryotes, like those of eukaryotes, must be carefully condensed to fit into and function within the cells they control. The resultant “nucleoid” structure can be partitioned into four macrodomains (MDs): Ori (containing the replication origin), Right, Left, and Ter (containing the replication termination site). This organization is critical for chromosome segregation during cell division.

Dupaigne *et al.* analyzed the structure and function of the Macrodomain Ter protein (MatP) from *Escherichia coli* and *Yersinia pestis*, which

is specifically responsible for Ter MD organization. MatP was composed of three domains. The central domain was required for MatP dimerization. The N-terminal four-helix-bundle domain bound specifically to a DNA consensus sequence, *matS*, scattered throughout the Ter MD but was not found in any of the other MDs. The C-terminal coiled-coil domain drove association of the DNA-

bound MatP dimers into tetramers. The tetramer formed a long rod-shaped complex, with the two DNA binding sites at opposite ends, up to 135 Å apart. The coiled-coil domain could also kink to impart a variety of bend angles to the rod complex and was critical for condensation of the Ter MD DNA, probably through MatP's ability to organize DNA between distant *matS* sites into loops. — GR

Mol. Cell. **10.1016/j.molcel.2012.09.009** (2012).



recorded history—and the resulting devastation after both the earthquake and the subsequent tsunami demonstrated that the model used to assess risk in that region may need serious revision. However, there were some signs that the region had the potential for a very large earthquake. Previous, although uncertain, historical accounts and sedimentological records of a large tsunami point to the

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(and 3 you probably
shouldn't) know
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Virginia Lee, Univ. of Pennsylvania
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Olle Lindvall, Univ. Hospital, Lund
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Jonathan Losos, Harvard Univ.
Ke Lu, Chinese Acad. of Sciences
Christian Lüscher, Univ. of Geneva
Laura Machesky, CRUK Beatson Inst. for Cancer Research
Anna Magurran, Univ. of St. Andrews
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Janice Orin, Univ. of Minnesota
Christine Ortiz, MIT
Andrew Oswald, Univ. of Warwick
Steve Palumbi, Stanford Univ.
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Donald R. Paul, Univ. of Texas at Austin
P. David Pearson, Univ. of California, Berkeley
Reginald M. Penner, Univ. of California, Irvine
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Jens Rostrup-Nielsen, Haldor Topsøe
Mike Ryan, Univ. of Texas, Austin
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Miquel Salmeron, Lawrence Berkeley National Lab
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Randy Seeley, Univ. of Cincinnati
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The Dreyfus Prize in the Chemical Sciences



The Dreyfus Prize in the Chemical Sciences recognizes an individual for exceptional and original research in a selected area of chemistry that has advanced the field in a major way. The Prize is open to individuals, both domestically and internationally.

The 2013 Dreyfus Prize will be awarded in the field of:

chemical instrumentation

The Dreyfus Prize, awarded biennially, consists of a citation, a medal, and a monetary award of \$250,000.

The nomination deadline is March 1, 2013. For further information, see www.dreyfus.org.

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LOCATION: Jackson
Park Health Club

ARTICLE: *An Electronic
Second Skin*

DATE: Sep 21, 7:43am

LOCATION: University
Faculty Lounge

ARTICLE: *The Visual
Impact of Gossip*

DATE: Sep 21, 4:22pm

LOCATION: Hemlock Bar

ARTICLE: *Quantum
Simulation of Frustrated
Classical Magnetism in
Triangular Optical Lattices*

DATE: Sep 21, 9:21pm

LOCATION: Gyro King

ARTICLE: *Cavemen
Craved Carbs, Too*

DATE: Sep 21, 1:13pm

LOCATION: Bed

ARTICLE: *Consciousness:
What, How and Why*

DATE: Sep 21, 10:56pm



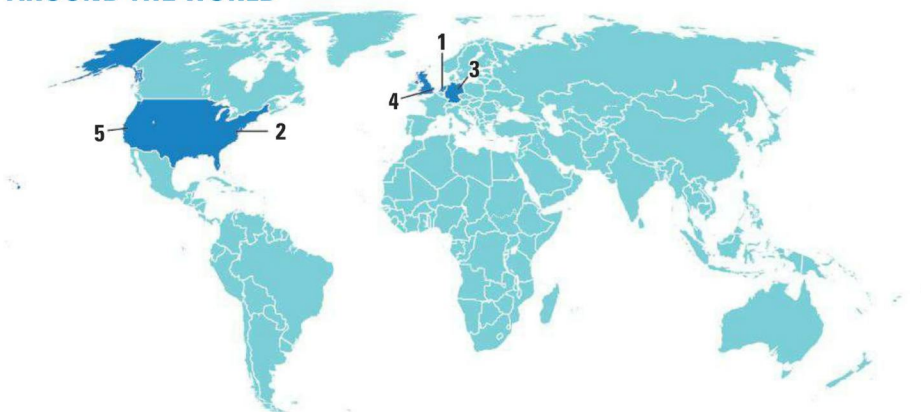
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AROUND THE WORLD



The Hague 1

A Place for All at The Climate Science Table

The Dutch government has created an online forum where “climate experts representing the full range of views” can discuss the hottest topics in climate change. The public can provide running commentary.

The site, launched this week at ClimateDialogue.org, began by tackling Arctic sea ice. Three respected American climate



scientists will debate the causes of sea-ice decline—global warming and natural variability lead the list—and when the Arctic Ocean could be ice-free. The scientists’ estimates “range from 2016 to the end of the 21st century, or even later,” a Climate Dialogue statement said.

The spread of opinion is deliberately broad. ClimateDialogue.org is one of several government projects the Dutch parliament has requested to “involve climate skeptics in future studies on climate change,” another statement explained. An advisory board of six scientists and a retired business executive will advise an editorial staff and “guard the neutrality of the platform,” the site says.

Washington, D.C. 2

USAID Launches New Science Program

The U.S. Agency for International Development (USAID) last week announced major awards at seven universities in the United States and abroad to support “development labs” that will design innovative, low-cost approaches to improving health and reducing poverty and conflicts. The program, called the Higher Education Solutions Network, intends to invest \$130 million over 5 years.

Each of the seven institutions will receive grants of up to \$5 million a year, with a 60% required match by the universities. As an example of the kinds of projects the program wants to encourage, USAID Administrator Rajiv Shah cited a cheap bucket filter designed at the Massachusetts Institute of Technology (MIT) in Cambridge to remove arsenic from groundwater in Bangladesh.

Pedro Sanchez, of the Earth Institute at Columbia University, says the concept of the awards is very innovative. “It’s a great step forward,” he adds.

The winners are Texas A&M University; Makerere University in Uganda; MIT; Michigan State University; Duke University; the University of California, Berkeley; and the College of William & Mary. USAID intends to call for more proposals in the future.

<http://scim.ag/USAIDprgms>

Berlin 3

Biomedicine Gets Boost

Two of Berlin’s largest biomedical research centers are joining forces to form the Berlin Institute of Health (BIH), a cooperative venture that will receive at least €320 million in new funding over the next 5 years. The union of the Max Delbrück Center for Molecu-



Charité campus

lar Medicine and the Charité, the university clinic of Berlin’s Humboldt University and Free University, will help attract top clinicians, researchers, and medical students to Berlin, says the city’s mayor, Klaus Wowereit.

The new institute’s organization will take shape next year, says Charité head Karl Max Einhäupl, with an international evaluation team advising BIH on how to best combine the two organizations’ strengths.

BIH bends some of the strict German rules about what types of research organizations the federal government can fund. Universities are primarily funded by the *Länder* (states). In this case, however, the federal government will provide 90% of the €300 million budgeted for BIH between 2013 and 2018. The privately financed Charité Foundation has promised an additional €40 million over 10 years. <http://scim.ag/Berlinbiomed>

London 4

Launching New Money Into Space

In a speech given on 9 November at the Royal Society, Britain’s chief finance minister, Chancellor of the Exchequer George Osborne, announced that the government would increase spending on space by 30%, or £60 million per year.

The move comes just days before European ministers responsible for space decide the European Space Agency’s (ESA’s) budget and spending priorities for the next 4 years. The extra money should allow Britain’s ESA delegation to make more substantial commitments to agency projects, such as the



Osborne

ExoMars project to send landers and orbiters to the Red Planet. NASA withdrew from ExoMars earlier this year, and ministers from ESA's 19 member states must decide at the meeting whether to form a new collaboration with Russia or go it alone.

Although the chancellor's announcement boosts Britain's ESA contribution to about €300 million annually, the United Kingdom still contributes less to ESA than Italy (€350 million in 2012), France (€751 million), and Germany (€714 million). <http://scim.ag/Moneyinspace>

Sacramento 5

Rejecting GM Food Labels, Backing Education

After months of heated public debate and more than \$50 million in campaign spending, Californians on 6 November voted against Proposition 37, which called for the mandatory labeling of genetically modified (GM) food sold in the state. Although early polls showed considerable public support for GM food labeling, 53% of voters ended up rejecting the measure.

Golden State voters also backed Governor Jerry Brown's plan to save schools from drastic budget cuts. Proposition 30, supported by 54% of voters, will raise the state income tax for wealthy residents for 7 years and increase California's sales tax by 0.25%. The measure is expected to provide an estimated \$6 billion annually for K–12 education and community colleges, and prevent additional cuts and tuition hikes at state universities. <http://scim.ag/GMlabel>; http://scim.ag/_HigherEd



NOTED

>“I give a shit, do you?” is the theme of this year's World Toilet Day (www.worldtoiletday.org), held annually on 19 November. Organized by the Water Supply and Sanitation Collaborative Council and the World Toilet Organization, the event aims to raise awareness of the 2.5 billion people around the world living without proper sanitation.

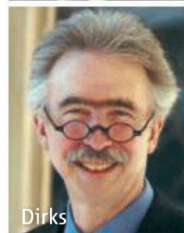
NEWSMAKERS

Researchers to Head Yale University and UC Berkeley

This month, psychologist **Peter Salovey** and anthropologist **Nicholas Dirks** were picked to lead Yale University and the University of California (UC), Berkeley, respectively.

Salovey, 54, is well-known for advancing studies of “emotional intelligence,” described as the ability to monitor one's own and others' feelings, discriminate among them, and use this information to guide thinking and action. The work has been used to alter behavior, particularly in AIDS prevention, says Alan Kraut, executive director of the Association for Psychological Science. Salovey, currently Yale's provost, succeeds President Richard Levin on 30 June 2013.

Dirks, 61, currently Columbia University's executive vice president for arts and sciences and dean of the faculty, has done field research in India and Britain and has authored three books on Indian culture and history. Nominated to succeed Robert Birgeneau as Berkeley's chancellor on 1 June 2013, Dirks is expected to be approved by the UC Board of Regents later this month.



Darwin for Congress?

Charles Darwin won support from at least 4000 voters in the 10th congressional district of Georgia thanks to an initiative headed by **James Leebens-Mack**, a plant biologist at the University of Georgia in Athens.



Leebens-Mack was deeply troubled by a speech that Representative Paul Broun (R-GA), who represents the 10th district in the U.S. House of Representatives, gave at an Athens church in October. Broun derided teachings on evolution, embryology, and the big bang theory as “lies straight from the pit of Hell.” Broun, a medical doctor, serves on the House Committee on Science, Space, and Technology.

Leebens-Mack channeled his outrage by

THEY SAID IT

“He is the latest in a long line of rulers and important people who have hired or been approached by scientists and physicians who claim to have found some kind of elixir capable of prolonging life.”

—Jennifer Rampling, a science historian at the University of Cambridge in the United Kingdom, in *The Telegraph* on reports that Kazakhstani scientists are developing a life-extending yogurt-based drink for the country's leader, Nursultan Nazarbayev.

setting up a Facebook page inviting citizens to vote for Darwin as a write-in candidate against Broun—who ran unopposed. Broun won comfortably, but Darwin got enough votes that Leebens-Mack says it's clear many people in the district were “not happy with antiscience statements.”

Would Leebens-Mack run against Broun himself in 2014? “I enjoy my job as a plant biologist,” he says. “It would be too big a sacrifice to give that up to run for Congress.” <http://scim.ag/Darwinrep>

Inaugural Picks for John Maddox Prize

A Chinese science reporter and a British psychiatrist are the first to win an award, named for former *Nature* editor John Maddox, honoring people who have defended sound science in the face of strong opposition. The prize, sponsored by *Nature* and the Kohn Foundation and organized by the U.K. charity Sense About Science, comes with £2000 for each winner.

Fang Shi-min, a biochemist-turned-science journalist, won the award for exposing scandals in Chinese science and medicine on his Web site New Threads. Fang attacked supposedly rejuvenating but unproven DNA supplements and took aim at traditional Chinese medicine (*Science*, 8 February 2008, p. 709). In 2010, thugs hired by a urologist whom Fang had criticized attacked him with a hammer.



>>

>>NEWSMAKERS

Simon Wessely of King's College London, the other winner, says he suffered intimidation—including death threats—



Wessely

and harassment for his research on chronic fatigue syndrome (CFS). Wessely demonstrated a big overlap between CFS and clinical depression and pioneered cognitive behavioral therapy as

a CFS treatment, angering patient advocates who see CFS as purely physical. "I'm pleased that people recognized the pressure brought to bear on me and many colleagues," he says.

FINDINGS

I Scratch, You Itch

Seeing someone yawn or hearing someone laugh makes you likely to follow suit. The same goes for scratching an itch. Now, for the first time, researchers have investigated the neural basis of contagious itch, identifying several brain regions whose activity predicts how susceptible people are to feeling itchy when they see someone else scratch.

Researchers in the United Kingdom showed volunteers video clips of people scratching an arm or a spot on their chest.

Sure enough, subjects reported feeling more itchy, and most scratched themselves at least once during the experiment.

When a subset of the volunteers watched the videos inside a functional magnetic resonance imaging scanner, the scans revealed activity in several of the same brain regions known to fire up in response to an itch-inducing histamine injection. Activity in three of these areas correlated with subjects' self-reported itchiness, the team reported online on 12 November in the *Proceedings of the National Academy of Sciences*. Personality tests suggested that the trait that best predicts susceptibility to contagious itch is neuroticism, not empathy, as some researchers have suggested. http://scim.ag/_itch



Protein Makes Sperm Flee

A compound that causes immature sperm cells to flee the testes early may provide new leads for contraceptives.

Scientists pursuing a "male pill" have recently found multiple ways to disrupt sperm production, usually by shutting down genes and proteins unique to the tes-

tes (*Science*, 19 October, p. 318). Now, a team led by C. Yan Cheng of the Population Council's Center for Biomedical Research in New York City has identified a new way to stop spermatogenesis: disrupting the blood-testis barrier, a cellular firewall between the testes and blood circulation.

When Cheng's team injected a special protein fragment into rat testes, the blood-testis barrier broke down. This caused immature sperm to drift out of the testes early, before they were capable of fertilizing eggs. More importantly, these changes were reversible. The team reported their findings on 13 November in *Nature Communications*.

Any potential male contraceptive would be many years off and would require many more tests. Cheng's team, for instance, has not tested whether rats injected with this protein fragment father fewer offspring. But Cheng says the advantage of this protein over other potential contraceptives is that the body produces it naturally in small amounts, so it's likely to be well tolerated. <http://scim.ag/spermflee>

Science LIVE

ScienceLive returns on Thursday, 29 November, at 3 p.m. EST for a chat on the imminent "end of the world." <http://scim.ag/science-live>

Random Sample

Cellular Chaos on the Dance Floor

Molecules in a cell move and run into each other all the time. Now, a scientist and a dancer have figured out a way to model that movement—using people.

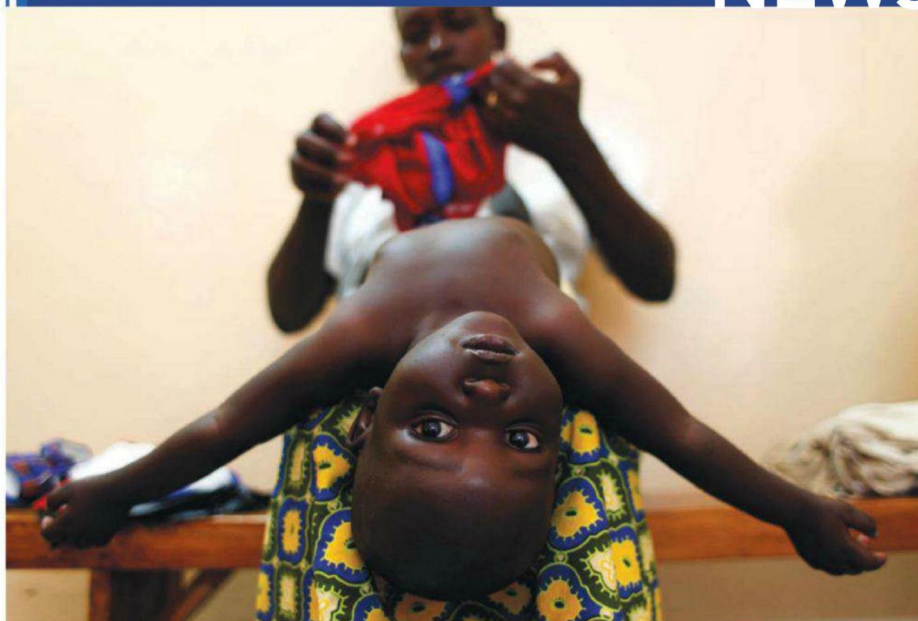
David Odde, a biomedical engineer at the University of Minnesota, Twin Cities, and Carl Flink, artistic director of the dance company Black Label Movement and head of the university's department of Theatre Arts and Dance, originally got together with the idea of presenting Odde's research in dance form: specifically, how the molecules that comprise part of a cell's internal skeleton come together and fall apart. Flink worked out how dancers could slam into each other safely—inflatable "sumo suits" and body armor were initially considered—and follow rules in a way that approximates randomness.

But the company has never performed these routines for audiences; instead, Flink and Odde use "bodystorming" to quickly try out hypotheses explaining molecular movements within cells—Odde has already rejected one idea he had proposed. "By giving specific instructions to dancers, you are forced to articulate what your hypothesis is," he says. Programming computer simulations can take months. With dance, "we immediately get to see it play out." The collaboration has been so successful that Odde and



Flink hold weekly joint lab meetings in a dance studio and published their work online on 1 November in *Trends in Cell Biology*.

"We as scientists spend a lot of time thinking," Odde says. But, when he, his students, or other scientists get into the dance, "you suddenly feel like you're in that space. You can feel it kind of in your gut, what it means to be a molecule." <http://scim.ag/bodystorm>



Vulnerable. A mother dresses her baby, a participant in the RTS,S trial in Kombewa, Kenya.

GLOBAL HEALTH

Disappointing Results Blunt Hopes for Malaria Vaccine

Scientists have released new trial results from the most advanced candidate vaccine for malaria, now in development for more than 2 decades, and there's no way around it: They're very disappointing. The data show that the vaccine lowers the risk of clinical episodes of malaria by just 31% among babies who receive their first dose between 6 and 12 weeks of age.

The results, presented on 9 November at the International African Vaccinology Conference in Cape Town and published online by *The New England Journal of Medicine*, dampen the hope that the vaccine, called RTS,S, might be used as part of babies' routine vaccinations in the first months of life. That would have been by far the easiest way to administer it, because the public health infrastructure is already in place.

It now seems unlikely that the vaccine will be approved for wide use by 2015, as scientists had hoped. Still, researchers have vowed not to give up on RTS,S; GlaxoSmithKline (GSK), which developed it in partnership with the PATH Malaria Vaccine Initiative (MVI), "remains completely committed" to further developing it, the company's CEO, Andrew Witty, said last week at a press conference.

In 2011, researchers presented data from the same trial that showed that RTS,S reduced the risk of malaria in children who received their first dose of the three-shot vaccine

between 5 and 17 months of age by 56%, a result in line with previous studies (*Science*, 21 October 2011, p. 298). The new results "leave us in an uncomfortable position," says co-author Pedro Alonso of the Barcelona Institute for Global Health in Spain. The vaccine may provide significant benefits when given to older babies and toddlers, but administering it to them would be logistically difficult and more expensive.

RTS,S is an engineered protein combining a protein fragment from *Plasmodium falciparum*, the malaria parasite, and a protein from the hepatitis B virus aimed at bolstering the body's immune response. The trial, the first-ever phase III trial of a malaria vaccine, is still ongoing, and full data won't be ready until late 2014. Those results should also show whether a booster 18 months after the third shot affects the vaccine's performance, and how long protection lasts.

The final results might help explain the differences between the age groups, says Salim Abdulla, director of the Ifakara Health Institute in Tanzania, one of the study sites. Possible explanations include the younger babies' less-developed immune systems, interference from maternal antibodies that babies carry in their first months of life, and interactions with other vaccines administered at the same time.

There may also be differences in the vaccine's efficacy in areas with different levels

of malaria transmission. A phase II study of RTS,S in infants showed much higher efficacy, but that was in three sites with moderate malaria transmission, notes Joe Cohen, one of the vaccine's inventors and an adviser to the GSK Malaria Vaccine Project. The phase III trial, which involves 15,460 children at 11 sites in seven sub-Saharan African countries, includes areas with much more malaria.

"We'd like to have seen higher efficacy than we've seen" in infants, Witty acknowledged last week. "It's a little frustrating that we're seeing different protection in different age groups. But that's not surprising, and it's not the first time that we've seen different immunological responses in different patient groups."

Indeed, for scientists familiar with last year's data, the results were "about what was expected," says Christopher Plowe, a malaria vaccine expert at the University of Maryland School of Medicine in Baltimore, who is not involved in the trial. (Last year's study included a preliminary estimate of efficacy for all children, which was lower than that for toddlers.) The result "makes it harder to envision adding this vaccine to the [standard vaccination] schedule," Plowe says. "At best we have one more modestly efficacious malaria-control tool" that could help in some places under some conditions. "If it were any other disease, we would probably be saying that this vaccine doesn't work," he says.

Whether and how RTS,S might ultimately be used "depends on the cost-effectiveness analysis and donor enthusiasm," says Nicholas White, a malaria expert at Mahidol University in Bangkok. GSK has promised to sell the vaccine at the cost of manufacturing plus 5%, which will be reinvested into a fund for research into vaccines against malaria or other neglected tropical diseases.

Amid the gloom, Abdulla and Cohen say they are encouraged by the vaccine's safety. Although babies developed a mild or moderate fever 31% of the time after receiving a dose of the vaccine (compared with 21% among those who received the control vaccine against meningococcal disease), the rate of severe side effects was slightly lower than in the control group.

Alonso, on the other hand, warns that some trends in the data suggest more bad news may be coming. There is "strong evidence," he says, that the vaccine offers much higher protection in the first months following the proto-

col's three-shot series than later on, in both age groups. "If you're only protecting ... for a few months, that's not so helpful," he says.

"There are promising early clinical and preclinical results" from vaccines that use different *P. falciparum* protein fragments, Plowe says, and many hope that combining

those with RTS,S might prompt better protection. An unconventional vaccine based on the whole parasite, grown in mosquitoes and killed by radiation, is also moving forward; Sanaria, the company developing it, is preparing to start trials in Africa, CEO Stephen Hoffman said at a meeting in

Basel, Switzerland, last month.

But those alternatives are far behind RTS,S. "No other candidate has actually shown efficacy in Africa," Alonso says. "Any other vaccine is at least 10 years away."

—GRETCHEN VOGEL

With reporting by Leslie Roberts.

EUROPE

Researchers Lobby to Spare Science From E.U. Budget Cuts

If you cut, don't cut research. That's the message from an increasingly loud chorus of scientists and research organizations in the run-up to major negotiations about the European Union's budget for the remainder of the decade. On 22 and 23 November, the heads of the European Union's 27 member countries will meet in Brussels to hammer out the union's overall budget from 2014 through 2020. The ongoing, severe economic crisis in Europe has prompted calls to cut the proposed €1 trillion E.U. budget by €50 billion or more. That threatens the proposed €80 billion budget for Horizon 2020, the program that will fund research and innovation across the European Union for those 7 years.

European scientists and their supporters are now making the case that research should

Germany's research and education minister, Annette Schavan, said at a meeting in Berlin last week. "I will personally champion this cause."

In June 2011, after hard lobbying by Research Commissioner Máire Geoghegan-Quinn, the European Commission—the union's executive arm—proposed spending €80 billion on Horizon 2020, a 60% increase over Framework 7, the current 7-year plan, which ends in 2013. The proposal includes, for example, 6% yearly budget increases for the European Research Council (ERC), which funds basic research by top researchers. The European Parliament was even more generous in its proposal, allotting Horizon 2020 €100 billion.

But the budget has to be agreed to by the E.U. member countries as well, and many of their leaders say the union has to tighten its belt just like everybody else. The Parliament's largesse for Horizon 2020, unlikely from the start, now seems impossibly optimistic, observers say, and even the commission's €80 billion plan may not make it. "I have heard speculation ranging from 40 billion to 70 billion," says ERC President Helga Nowotny.

Even €80 billion would be less of a boost than it appears to be, says Christopher Hull, secretary general of the European Association of Research and Technology Organizations. Framework 7 funding has ramped up each year, and the program will spend roughly €10 billion annually in 2012 and 2013. So flat budgets through 2020 would actually require at least €70 billion overall, Hull says: "If we get 60 billion, ... then what the commission has proposed no longer works." Finding €20 billion worth of cuts in Horizon 2020 will require a major reorganization, he predicts: "It's going to be an unholy mess."

Cyprus, which is one of the European Union's smallest countries but currently holds the rotating 6-month European presidency, will chair the summit next week. As a starting point for negotiations, it has proposed an overall €50 billion cut to the commission's spending plan, with 4% shaved off the budget for "Competitiveness," which includes Horizon 2020. The final cuts will be larger, the document warns, because "[m]ore sizeable reductions are needed in order to reach a compromise." British Prime Minister David Cameron has said that he will veto any increase in the overall E.U. budget from current levels; a group of countries including Germany is calling for a slight decrease from the commission's proposal, while the 12 newest E.U. members—which stand to benefit most from a hike—have joined the European Parliament in calling for a substantial increase over current spending.

However, some countries calling for the most austerity also advocate spending more on research. Sweden, for example, favors overall cuts of more than €150 billion but wants a bigger part of the E.U. budget spent on research and innovation. "Cutting the budget doesn't mean that you can't also reform the budget," says Daniel Wennick of the business group the Confederation of Swedish Enterprise. The Swedes say that the European Union should instead trim its generous agricultural subsidies, which France strongly opposes. Europe's leaders may well fail to reach a deal at the summit next week, and a final decision isn't expected until summer 2013.

Still, some observers are cautiously optimistic that Europe's leaders realize the importance of research for Europe's economy. "Spending on research makes good sense—good economic sense and good sense for the future for Europe," says Stephan Kuster, head of policy affairs at Science Europe, which unites national research funding organizations. "We think people are starting to listen."

—GRETCHEN VOGEL



Downsizing. European Research Commissioner Máire Geoghegan-Quinn may see the proposed €80 billion budget for Horizon 2020 slashed.

be spared the trimming and squeezing. More than 130,000 researchers around the world have signed an online petition in support of a letter penned by 44 European Nobel laureates and six Fields medalists asking politicians to consider "what will be the role of science in Europe's future?" If research budgets are significantly cut, the letter warns, "we risk losing a generation of talented scientists just when Europe needs them most."

Leading research organizations have added their voices, as have some politicians. "Even now, in difficult times, Europe can, with the right decisions for research and development, tap new sources of strength,"



RESEARCH PHILANTHROPY

Billionaire Restores Funding For Novel Biomedical Network

Bruce Dobkin isn't much of a gambler. But the neurologist at the University of California, Los Angeles (UCLA), is among those rolling the dice on an unusual strategy to fund medical research.

In October, Dobkin spent 2 days at The Venetian, a sprawling hotel and casino on the Las Vegas Strip, to help resuscitate a research collaboration funded by the casino's billionaire owner, Sheldon Adelson. In 2006, Adelson, now the 12th richest person in the United States according to *Forbes*, started the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (AMRF) along with his physician wife. And as the foundation's executive director for its first 18 months, Dobkin made twice-yearly visits to the casino to help oversee its research strategy. But the foundation almost collapsed after the 2008 recession took a huge bite out of Adelson's company, Las Vegas Sands Corp.

Now that its share price has bounced back, Adelson wants to reenergize the scientific network. AMRF has no fixed budget, but this year it's putting roughly \$20 million to \$25 million into bold ideas in neuroscience and cancer. And Adelson hints that number may grow.

Adelson has been a lightning rod for controversy over the years. He's engaged in numerous legal clashes with business competitors and unions. A 2011 earnings report

filed by Las Vegas Sands reported that the U.S. government was investigating the company for possible violations of the Foreign Corrupt Practices Act, 4 years after it opened the largest casino in the world in Macao.

But those controversies haven't stopped Adelson and his wife Miriam, an Israeli physician with an interest in studying drug addiction, from making sizable donations to their favorite causes. *The New York Times* has called Adelson the "biggest single donor in political history," giving tens of millions in the most recent election cycle to Republican candidates, most of whom lost—including Republican presidential nominee Mitt Romney. Adelson is also a big supporter of Israel and started a free newspaper to help promote the policies of his friend, Israeli Prime Minister Benjamin Netanyahu. It is now Israel's most read paper.

When *Science* asked him how he balances

In good health. The Adelsons have revved up their medical research foundation.

his various causes, he didn't mince words. "Have you seen my name listed in *Forbes*?" he asked. "There's plenty to go around."

Adelson hatched the idea for his foundation when seeking treatment from Dobkin for a progressive weakness in his right leg. The two began discussing "the status of medical research, difficulties of funding, funding models, the silos that might inhibit people from sharing work," Dobkin says.

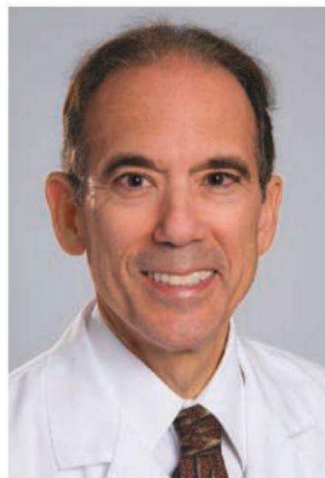
One day Dobkin arrived late for an appointment and Adelson told him he looked "beat-up." Dobkin explained that he'd been racing to meet a deadline for grant applications to the U.S. National Institutes of Health (NIH). That led to a conversation about how the federal government funds biomedical research.

Adelson was dumbfounded. Scientists came up with their own ideas, rather than being told by NIH what to do? The work had to be relatively incremental to garner support? No doubt there was a better way, he thought: Gather the top experts in a field and instruct them to solve the problem, together.

The counterargument "is that no one ever funds that way," Dobkin says. "Would scientists work together with each other? Would IP [intellectual property] issues arise? ... Who would be the first author on the paper?"

Adelson wasn't deterred. Let's do it, he told Dobkin, who began enlisting specialists from many subfields, including genomics, informatics, cell biology, and translational research. Adelson also had an interest in certain cancers; he'd battled melanoma, and his sister had died of ovarian cancer. So Dobkin reached out to UCLA colleague Dennis Slamon, chief of hematology/oncology, to create teams in ovarian and other cancers, while Adelson asked Donald Morton at the John Wayne Cancer Institute in Santa Monica, California, to spearhead a melanoma initiative.

The neural repair group was the first to meet in The Venetian, back in 2006. "People began feeling each other out," Dobkin says. "At the end of the meeting Sheldon and Miriam turned to me and said, 'Do you think they buy into it?'" Dobkin recalls. "I looked around the room and in all four corners ... were groups of people working together, drawing on a napkin." He felt hopeful.



Team player. Bruce Dobkin helped create innovative collaborations.

The group expanded. Its members decided how to allocate Adelson's money among them, disbursing grants of between \$125,000 and \$400,000 per lab. By September 2008, the semiannual gathering had grown to 167 investigators from 65 institutions.

Simultaneously, the stock price of Las Vegas Sands began tanking. From \$46 per share in September 2008, it plunged to about \$3 in late November. Adelson fought to keep the company from going bankrupt. "You could see the writing on the wall" regarding the foundation's future, Dobkin says.

"We went into what I call a maintenance mode," Adelson says. Funding stopped except for about 55 postdoctoral fellowships, at about \$40,000 each per year.

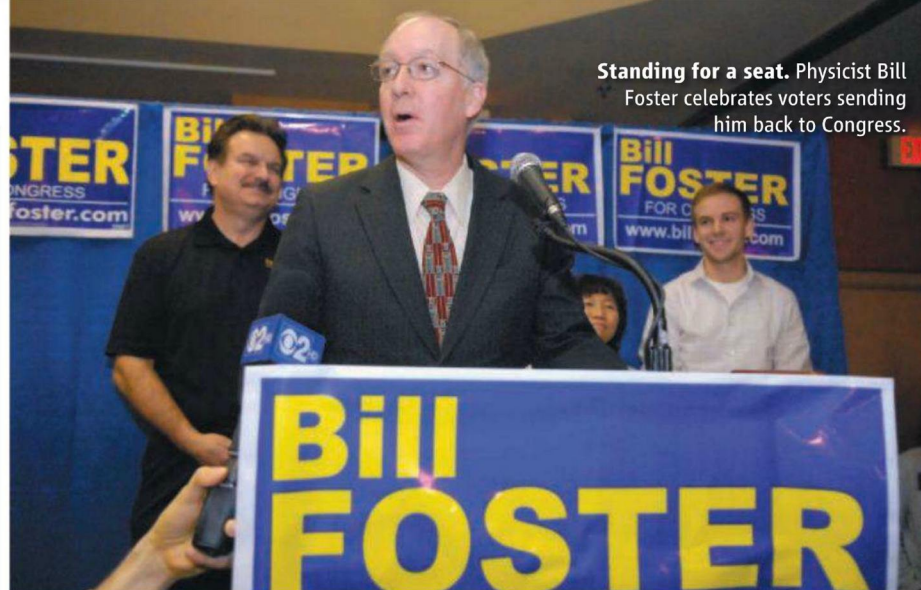
By last year, however, Las Vegas Sands had recovered, and it's currently worth about \$36 billion. Adelson was ready to get his medical foundation started again. Chief Science Officer Kenneth Fasman was charged with informing the researchers that new money would start flowing, thereby putting Adelson's model to the test.

"Some people express a lot of discomfort" with this approach, Fasman says. One concern is winding up with a "pack mentality, which takes the whole group completely in the wrong direction." A safeguard, he says, is the quality of participants and the fact that the work is presented at public meetings and published. Fasman acknowledges that "this model may feel a bit clubby or introverted," but he says that the Adelsons "want to let it run for a little while and see if it has significant impact."

Last month's meeting allowed the scientists to hash out the details of the next 2 years of collaborative experiments. "There's no question that people are taking on projects that they might never have otherwise done," says Dobkin, who is part of a group studying how to improve learning and memory after brain injury. Among other challenges are overcoming resistance to the new generation of melanoma drugs, exploring the interface between the ubiquitin pathway and DNA damage, and regenerating the optic nerve.

In addition to nurturing scientific breakthroughs, Adelson hopes that the foundation will win him the respect of those who oversee biomedical research funding. "I know a lot of people in Congress," he says. But he thinks he can gain "more credibility" by having "150, 200 people working on" AMRF-funded collaborations. And with his company back on firm ground, he says he's in it for the long haul.

—JENNIFER COUZIN-FRANKEL



Standing for a seat. Physicist Bill Foster celebrates voters sending him back to Congress.

U.S. ELECTION

Old Challenges, New Faces Await Science Community in 2013

The American people may have voted for political continuity in retaining President Barack Obama and preserving a divided Congress. But that doesn't mean the next 2 years will be status quo for science.

When the new Congress convenes in January, the committee that keeps the closest eye on federal science policy—the Committee on Science, Space, and Technology in the House of Representatives—will have a new chair. The Republican lawmaker who gets the job (see sidebar) is expected to reinvigorate the panel as it tackles two major pieces of legislation. The full House will be without two of its staunchest advocates for federally funded research, as veteran representatives Judy Biggert (R-IL) and Brian Bilbray (R-CA) lost their seats. Partially offsetting those losses, the Democrat who beat Biggert, Bill Foster, is a high-energy physicist turned politician who will be returning after a 2-year absence.

To be sure, those developments are less momentous than the looming threat of massive cuts to both civilian and military programs that tops the agenda for the lame-duck session of Congress that began this week. The so-called sequestration bomb, set to go off in January, would tear an 8% hole in the budgets of most research agencies.

Defusing it will require the type of bipartisanship between the Republican-led House and the Democratic-led Senate that has been

absent for the past 2 years. Still, there is hope that the two parties will agree to delay sequestration long enough to give the new Congress time to strike a more lasting bargain with the White House on how to shrink the \$1.3 trillion budget deficit.

In spite of the recent budget-cutting fervor, legislators have largely preserved funding for science. With sequestration looming, a key question will be whether some areas of science are likely to be sacrificed in the political horse trading that sequestration—or the deal that heads it off—will likely involve.

University lobbyists believe that Obama hasn't forgotten their cause. Last week, as he invited Republicans to the table for negotiations altering the tax code and reforming entitlement programs, Obama found room in the speech to once again emphasize the need to "give people the chance to get the education and training that businesses are looking for ... [and] make sure this country is a global leader in research and technology." Marvels Jennifer Poulakidas of the 218-member Association of Public and Land-grant Universities: "It's as if one of our members is writing his speeches."

In the meantime, the political show must go on. And that could mean a brighter spotlight on the House science committee, which for the past 2 years has been nearly invisible.

"I think it will be more active," predicts Representative Randy Hultgren (R-IL), who in his first term was recognized as a "champion of science" by the Science Coalition, an advocacy group of 51 research universities. "We were active, but I don't know if it was as purposeful and compelling as I would have liked it to be."

Online
sciencemag.org
Read interviews with Hultgren, Foster, Sensenbrenner, and Rohrabacher. http://scim.ag/election_2012

CREDIT: AP PHOTO/DAILY HERALD/LAURA STOECKER

Change Versus Experience?

This month, Republicans in the U.S. House of Representatives will choose from among a trio of veteran politicians vying to become the next chair of the House Committee on Science, Space, and Technology.

For one candidate—Representative F. James Sensenbrenner Jr. (R-WI)—it would be his second time around. For another, Representative Lamar Smith (R-TX), it would be a soft landing after party rules have prevented him from remaining atop a more prestigious panel. Both men say they have a history of working with Democrats to get things done.



Triple threat. Representatives Sensenbrenner (left), Smith (center), and Rohrabacher want to run the House science committee.

The third candidate, Representative Dana Rohrabacher (D-CA), says it is time for more pugnacious leadership on a panel that has become an afterthought in recent years. “If I’m chairman, the science committee will no longer be a backwater,” he promises.

The unusual three-way contest is the result of rules that are forcing

the current chair, Representative Ralph Hall (R-TX), to step aside after 6 years in leadership positions—4 years as ranking member and the past 2 as chair. Despite its grand title, the science panel has little authority over

the largest federal funder of research, the National Institutes of Health. But it does set broad policy for the National Science Foundation, NASA, the Department of Energy’s Office of Science, and other research agencies. And it can investigate questionable practices at those agencies.

All three candidates say that they would use those powers to highlight the importance of maintaining a strong basic research community, improving science education, and fostering technological innovation. Each sees renewing NASA’s authorizing legislation, which expires in 2013, as a top priority. And all are on record as doubting that human activities are contributing to global warming and opposing legislative efforts to reduce U.S. carbon emissions.

Given such similarities, Sensenbrenner, 69, says his experience—including 28 years on the science panel and the leadership of both the House Judiciary and science committees—should give him the edge. He says his biggest challenge will be figuring out how “to stretch the science research dollars at a time of obvious austerity.”

Smith, 64, also touts his record, which includes 26 years on the science committee, a stint as head of the Judiciary Committee, and a major role in writing a landmark patent law enacted last year. He supports allowing foreign-born scientists who earn their degrees in the United States to stay but has been unable to strike a deal with Democrats to get it done.

Rohrabacher, 65, says that 24 years on the committee have earned him a leadership role. Besides, he says, it’s not fair “one small clique of people should be transferring chairmanships among themselves.”

Issues other than the candidates’ positions on science may well determine the winner. Party leaders are also interested in which man will best promote the party’s agenda and raise money for the 2014 midterm election.

—DAVID MALAKOFF

One item on the committee’s agenda is a reauthorization of programs at NASA. That bill could test the Obama administration’s commitment to space science as the agency struggles to find money to complete work on a new rocket and capsule for human exploration.

The committee may also begin to update the America COMPETES Act, which expires next fall. First passed in 2007 and then extended in 2010, the legislation governs science and education programs at three key federal agencies—the National Science Foundation, the National Institute of Standards and Technology, and the Department of Energy (DOE). Among its many provisions is support for a 10-year doubling of those agencies’ budgets. That growth has not occurred, however, and the next version is likely to reflect the outcome of a broader discussion of federal support for basic research.

That debate will unfold minus Biggert, whose district includes DOE’s Argonne National Laboratory. Science lobbyists say that the moderate Republican’s voice on the committee will be sorely missed.

“In a time of fiscal restraint, it hasn’t been easy for a Republican House member to stand up for research and make it a priority, but she did,” says Barry Toiv of the 62-member Asso-

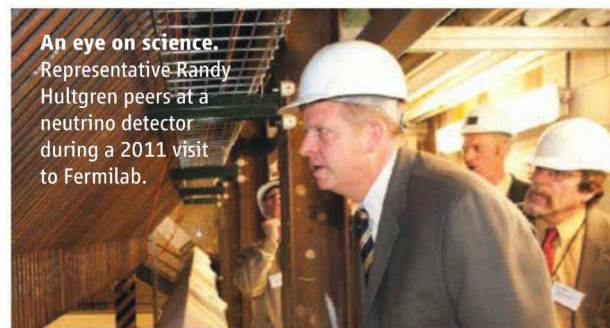
ciation of American Universities. Bilbray has been equally passionate about research as co-chair of the bipartisan Congressional Biomedical Research Caucus, notes Bill Andresen, head of federal relations for the University of Pennsylvania.

At the same time, the person who unseated Biggert, Democrat Foster, will double—to two—the number of Ph.D. physicists in the House. (Last week Representative Rush Holt [D-NJ], a former assistant director of DOE’s Princeton Plasma Physics Laboratory, was reelected to his eighth term.) Foster, who spent 22 years at DOE’s Fermi National Accelerator Laboratory (Fermilab), says he hasn’t decided whether to join the science panel.

In a previous 3-year stint in Congress, Foster focused his attention on banking reforms in the wake of the 2008 financial meltdown, and the economy remains his priority. “Given the number of physicists that were involved in the design of structured financial products, I think it’s only fair that physicists should contribute some effort to straightening out the mess,” he says.

Ironically, Foster lost in 2010 to Hultgren, who despite his lack of seniority hopes to be chosen to lead one of the science com-

mittee’s five subcommittees. Representing a different district that still includes Fermilab, Hultgren was reelected easily last week and wants to spread the “bug” that bit him after he landed on the science committee. One idea is to create a bipartisan national laboratory caucus in Congress to showcase



An eye on science. Representative Randy Hultgren peers at a neutrino detector during a 2011 visit to Fermilab.

research at all federal labs. And he thinks being a nonscientist “frees me up” to proselytize on behalf of the community.

“My constituent scientists are 10 times smarter than I am,” he says. “I can be their advocate and also help them to better explain to the public what they are doing.” If science ever needed advocates on Capitol Hill, now’s the time.

—JEFFREY MERVIS

With reporting by Adrian Cho.



Nearly Buried, Mussels Get a Helping Hand

North America is home to a record diversity of freshwater mussels with dazzling reproductive strategies and key ecological roles. But can they withstand the hard knocks of a modern world?

EVERY SPRING, A FRESHWATER MUSSEL called the snuffbox emerges from gravel stream bottoms for a violent bout of reproductive deception. The females have spent months buried in the sediment, brooding thousands of larvae that require a certain host to mature. Now the mussels lie on the streambed, their shells open wide. Playing dead, they wait for just the right fish to approach.

That fish, the logperch, spends its days hunting for insect larvae and fish eggs, rummaging under small stones and empty shells. When a logperch pokes its snout inside a snuffbox (*Epioblasma triquetra*), the mussel snaps shut. The fish is trapped between the serrated edges. For other fishes, this mistake would be fatal, but the logperch has a reinforced skull. As the fish struggles, the mussel pumps out its larvae, which clamp their tiny shells onto the

filaments of the logperch's gills. Then the mussel lets go. After several weeks of hitchhiking, the juvenile mussels drop from the gills and settle into their new habitat.

This aggressive tactic, discovered in 2003, is just one of the remarkable behaviors that freshwater mussels use to reproduce and spread upstream. Other species attract their fish hosts with lures that resemble fish eggs, crayfish, or even swimming minnows. "It's some of the most amazing mimicry in the world," says restoration biologist Jess Jones of the U.S. Fish and Wildlife Service (FWS) in Blacksburg, Virginia. And it's a North American specialty: The continent hosts the world's great-

est known assortment of mussels, 297 species, more than two-thirds of which are concentrated in the southeastern United States. Some rivers have more species of

Strong mussels. Studying healthy habitats, such as Virginia's Clinch River, helps guide mussel recovery.

mussels than are found in all of Europe.

But freshwater mussels are in trouble. They are the most endangered group of organisms in the United States, with most of their river and stream habitats devastated by dams, pollution, and invasive species such as the zebra mussel. Thirty-five species have been declared extinct, others are likely gone, and more than 70 species are teetering on the brink. The snuffbox, for example, was put on the U.S. endangered species list this past February; biologists estimate its population has declined by 90% over the past century. This month, FWS added another eight mussel species to its list. "It's the biggest conservation crisis in the U.S. that no one talks about," says Paul Johnson, who directs the Alabama Aquatic Biodiversity Center in Marion.

Mussel conservationists are intent on fixing the problem. They've persuaded a few dam operators to modify their water releases to improve conditions for mussels. They have also helped restore water quality in important mussel habitats. Researchers, meanwhile, are trying to solve some puzzles in mussel ecology, figure out how to culture more kinds of endangered mussels in captivity, and ramp up restoration efforts. Time is running short, says Robert Bringolf of the University of Georgia in Athens, but "hopefully we have a chance to turn things around."

North America owes its astounding freshwater biodiversity in large part to unique geology, which has provided a stable environment that enabled mussels to thrive and diversify for 60 million years (see p. 877). Historically, expansive shoals of mussels served as habitat for other aquatic organisms. By filtering water, mussels move nutrients through the food web, supporting nearby terrestrial ecosystems as well. The result: Rivers and streams with a greater number of mussel species tend to have richer algal and insect communities than those with fewer species, Caryn Vaughn of the University of Oklahoma, Norman, and colleagues concluded in a paper published in *Ecology* last month.

People have also long benefited from mussels. Massive middens hint at the untold numbers harvested by Native Americans for food. In the mid-19th century, a bustling industry sprang up to collect mussel shells for making buttons. Nowadays, workers grind mussels into "seeds"—bits of shell—that are shipped to Asia and placed into Pacific marine oysters to create cultured pearls. Most scientists, however, don't think that these activities put many mussel species in jeopardy of extinction.

Online

sciencemag.org

S Hear a podcast at <http://scim.ag/pod6109> and see a video on mussel conservation at http://scim.ag/mussel_vid with the author.

What has caused serious harm is widespread fragmentation and loss of habitat. Mining and deforestation, which polluted streams and clogged them with sediment, were already problems by the late 19th century. The worst trouble started in the early 1900s, when engineers built locks and dams in large numbers. These efforts culminated in the gargantuan dams constructed across the southeastern United States by the Tennessee Valley Authority (TVA) in the 1930s and '40s. Most mussel species can't live in the slow, muddy water and silty bottoms of the reservoirs formed by these dams. Nor can half the fish species that mussels need as hosts for their larvae.

Sometimes extirpation is delayed. Populations can persist, because the adults live for many decades. They might seem healthy, but they can't produce young, impaired either by pollution or because the fish hosts are gone. "It's an insidious decline," says David Strayer of the Cary Institute of Ecosystem Studies in Millbrook, New York. The population crash can come "breathtakingly fast" once these older mussels start dying.

Researchers are discovering more about these invisible threats. Lab studies show that mussels are sensitive to a number of common but poorly regulated water contaminants, such as the surfactants in the common herbicide glyphosate. In a detailed field study, Strayer and Heather Malcom, also at the Cary Institute, found that the absence of juveniles was highly correlated with ammonia—likely



Gotcha! To spread its larvae, the oyster mussel (top) catches a logperch, while the broken-ray mussel attracts bass with a minnow-like lure.

from fertilizer or manure—in the sediment where the mussels burrow. Spikes in ammonia concentrations "may be responsible for widespread declines of freshwater mussel populations, especially in agricultural areas," they reported in *Ecological Applications* in September. Some ecologists suspect that the current level permitted in surface water by the U.S. Environmental Protection Agency is dangerous for mussels.

The accelerating disappearance of mussels "really is a strong statement about what we've done to rivers," Bringolf says. In the Mississippi River Basin alone, perhaps less than 10% of the original habitat of endangered mussels remains unaltered by dams.

Positive steps

The situation is not entirely bleak. Once researchers began to understand the threats facing mussels, they were able to argue for major modifications of dam operations—helped by the legal hammer of the Endangered Species Act. Two decades ago, for instance, researchers couldn't find any endangered Cumberland monkeyface mussels in Tennessee's Duck River, which is controlled by the 855-meter-wide Normandy Dam. One problem was chronically low oxygen levels in the river below the dam. In 1991, TVA began to address that issue by blowing air into underground sluices as they released water from the reservoir—essentially creating a giant bubbler. Within a decade, researchers counted tens of thousands of mussels throughout 50 kilometers of river. "It shows you that flow restorations work and that we can recover some of these animals," Johnson says.

Conservationists have also encouraged efforts to improve water quality. Federal and state programs pay landowners to use best management practices that can reduce polluted runoff, including planting trees along stream banks and fencing out cattle. Organi-

The Evolutionary Allure of Mussels

North America's marvelous variety of mussels is due to a fortuitous combination of geology and climate. In the southeast, where the diversity is highest, watersheds have remained essentially unchanged for millions of years. The many isolated rivers provided room for new species and constant flows that prevented extinctions, unlike many waterways in Africa and Australia that vanish in severe droughts. The southern United States also escaped glaciation—unlike the northern United States and Europe, which has only about a dozen species of mussels. Rivers in the tropics, meanwhile, tend to lack nutrients and have too much silt for many mussels.

Over the past decade, genetic studies have revealed that mussel diversity in North America is even higher than past studies suggested, says fisheries biologist Wendell Haag of the U.S. Forest Service in Oxford, Mississippi. Widely separated populations that were once lumped into a single species because of their similar shell morphology, for example, are now understood to belong to multiple taxa. That matters: "A lot of species that we thought were widely distributed will turn out to have very restricted ranges and be highly imperiled," Haag says.

The exotic lures that some of these mussels use to attract their fish hosts are apparently unique to North America, although mussel biology is much less known in Asia. How the mussel-fish collaboration evolved is a mystery. In 2008, Haag and M. Christopher Barnhart of Missouri State University in Springfield proposed a scenario in the *Journal of the North American Ben-*

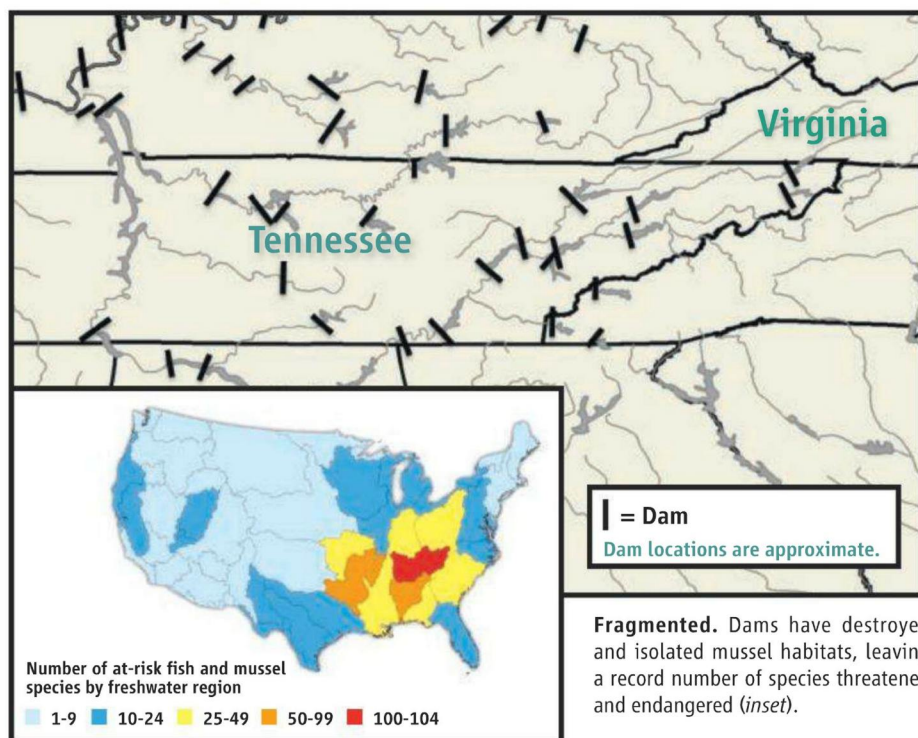
thological Society. Mussel larvae secrete threads, they noted, which help them stay afloat. Such threads may have become snagged on passing fish—giving some early mussels a benefit by carrying them into new habitat.

Another clever mussel reproductive strategy is producing larvae that appeal to hungry fish, giving the larvae a chance at hitching a ride on their gills. This approach may have started with ancestral mussels that, like some modern species, released millions of larvae without trying to attract a host fish. Over time, species that released their larvae in clumps called conglomerates, some of which are filled with colorful sterile eggs, may have gained an advantage because the clumps looked tasty.

The clumps may also have been a steppingstone to the more sophisticated strategy found in two groups of a diverse mussel tribe named Lampsilini. One group, which includes the fluted kidneyshell (*Ptychobranthus subtentum*), makes membrane-wrapped conglomerates that resemble fish eggs or insect larvae. Species in the second group, such as the orangenacre mucket (*Lampsilis perovalis*), spin out a "superconglomerate" that remains tethered to the mussel by a clear cord that is up to a meter long. It looks like a minnow swimming, complete with eyespots and countershading.

Some mussels have evolved even fancier lures by modifying their mantle. The rainbow mussel (*Villosa iris*) has one that resembles a crayfish, which it moves by rocking its shell back and forth. Wavy-rayed lamp-mussels (*Lampsilis fasciola*) each use one of three kinds of lures, perhaps to catch fish that have learned to avoid their neighbors. No matter, there's a sucker born every minute.

—E.S.



zations such as the Nature Conservancy have stepped in, purchasing land to protect important mussel habitat, such as the Clinch River in Virginia and Tennessee, and collaborating with farmers to restore it.

The most urgent work is to prevent the extinction of highly endangered mussels. Their small populations are vulnerable not only to chronic problems but also to freak accidents. For example, in the largest mass mortality recorded in the history of the U.S. Endangered Species Program, a 1998 spill from a chemical tanker truck killed more than 18,000 mussels in the Clinch River system. Some 750 of the dead were members of three endangered species: the tan riffleshell, the purple bean, and the rough rabbitsfoot. In 2003, the federal government settled with the trucking company for \$3.8 million in damages.

Relying in part on funding provided by such fines, biologists have established hatcheries for mussels—and their specialized fish hosts—to help boost wild populations. There are now 15 mussel farms, run with state and federal funds; the newest and biggest is the \$3 million Alabama Aquatic Biodiversity Center, which opened in 2010. This facility alone has the capacity to produce more than a million animals a year, which are used to establish new colonies—a kind of biological insurance policy against local disasters.

In late September, for example, a team from FWS and the Virginia Polytechnic Institute and State University (Virginia Tech)

in Blacksburg drove to the Powell River in Tennessee for their largest-ever release of oyster mussels and Cumberlandian combshells, two endangered species propagated in Virginia Tech's Freshwater Mollusk Conservation Center. Water quality in the Powell has slowly improved over the past decades, but 13 of the 35 species of mussels in the river are still endangered.

Working under the shade of hickory trees, field assistants in hip waders carried a large cooler full of mussels to the muddy riverbank. They splashed in and scattered several thousand juveniles. Meanwhile, lab manager Dan Hua carefully tucked 27 snuff-box mussels into the gravelly riverbed for the first time; the lab has only recently figured out how to culture this endangered species. "The most exciting thing is that our babies are home now," she said with a grin.

Boosting populations

Cultivating mussels is not easy, and it requires some sleuthing. If the preferred fish host is not known—often the situation with endangered species—researchers must become matchmakers, trying to find the right partners by testing various species in the lab. Next, researchers take female mussels into the lab, where they remove the larvae and then add them to the gills of host fish. Even more challenging is figuring out the right conditions for the subsequent care of the juveniles, Johnson says. In some systems, these 250-micrometer mussels can fall prey to tiny flatworms and other

predators in the tanks, with mortality rates of up to 80% or more in the first 2 months. Staff members must change the water and sediment regularly to prevent predators from booming. "It's like farming; you can't take a day off," Jones said recently, as he checked the gurgling tanks in his cavernous center.

Building on early work at Virginia Tech by fisheries biologist Richard Neves, researchers there and elsewhere have further improved propagation equipment and techniques. In the last 5 years or so, they have become adept at growing more and larger mussels. That's important because bigger juveniles have a higher chance of surviving in the wild. "It's opened up population restoration," says M. Christopher Barnhart of Missouri State University in Springfield, who has developed compact culture systems that deliver more feed to the juveniles and are widely used for rearing mussels. Larger mussels can also be tagged to better monitor success rates. Research at Virginia Tech by Hua and graduate student Caitlin Carey suggest that mortality rates in the wild for the bigger animals are less than 10% a year.

Two concerns with propagation, however, are unanticipated ecological impacts and inadvertent mixing of unidentified stocks; genetic background checks are just beginning. In addition, propagation, driven by the requirements of the Endangered Species Act, exclusively targets the most imperiled mussels. "The rarer a species becomes, the more attention it gets," Barnhart explains. That's why many biologists hope that common species can also be protected by better understanding and highlighting their benefits to ecosystems and humans. "The direction that we're trying to go in our research is to put monetary values on these services that mussels are providing," Vaughn says. For instance, mussels filter out phytoplankton, bacteria, and algae. That could be a good reason for a dam operator to keep water flowing in rivers during droughts, for instance, or to restore damaged rivers (so mussels can help keep them clean) rather than build an expensive new water treatment plant. It can be "cheaper to do the cleanup work than keep treating the water," Johnson says.

Whether or not mussels will ever be able to pay their own way to survival, their astonishing mimicry and reproductive talents inspire researchers to better understand and care for this hidden diversity. "Once I realized how highly evolved mussels really are, and that they are better at catching fish than I am, I didn't have anything but respect for them," Bringolf says. "This is what draws people in."

—ERIK STOKSTAD

CREDITS: J. JONES, U.S. FISH AND WILDLIFE SERVICE; (INSET) NATURESERVE/NATURAL HERITAGE NETWORK



CLIMATE CHANGE

Winds of Change

Antarctica's fate is not as simple as that of an ice cube melting in the sun, scaled up a trillionfold

PALMER STATION, ANTARCTICA—To Jennifer Blum, the empty nests on Torgersen Island are a harbinger of doom. The rugged outcrop is home to one of the largest colonies of Adélie penguins on the Antarctic Peninsula, a crooked sliver of land flanked by dozens of islands that reaches north toward the tip of South America. But the Adélies here are fading fast. There are fewer than 2000 breeding pairs, a quarter of the number found in the 1970s. Each spring, Blum, an ornithologist with Polar Oceans Research Group, a nonprofit in Sheridan, Montana, and her colleagues monitor colony size and breeding success. “You see a lot of newly abandoned nests every year,” she says. “The changes have been staggering.”

Blum's group has charted similar declines on nearby islands. She and others blame climate change. On the Antarctic Peninsula, the mean annual temperature is -3°C , more than 2° warmer than it was 50 years ago. Over the past 5 decades, winter temperatures have risen a staggering 6°C , or five times the global average. Roughly nine out of every 10 glaciers on the peninsula are retreating, says Hugh Ducklow, director of the Palmer Long-Term Ecological Research Station here on the

western coast of the Antarctic Peninsula. Coastal ice persists only 5 months a year, three months fewer than 30 years ago.

Warming has warped the peninsula's food web. But “global warming is only part of the story,” says David Thompson, a climate researcher at Colorado State University in Fort Collins. An unsung force now shaping the frozen continent's future is changing wind patterns. Retreating sea ice and stronger winds have caused seawater to mix more deeply, a process that churns sunlight-dependent phytoplankton into the ocean's depths. As a result, phytoplankton biomass has declined by 12% over the past 30 years. Higher on the food chain, that means fewer krill and fish larvae. These creatures are also getting hammered by the loss of sea ice, which hides them from predators.

More crucially, the complex interplay between air, sea, and ice has emerged as a central theme underlying climate change in Antarctica. Shifting wind patterns and corresponding ocean changes can explain climate responses across the continent. “It's quite a fabulous piece of climate science,” says Robert Bindaschadler, a glaciologist at NASA Goddard Space Flight Center in Greenbelt, Maryland. “It shows how the

Vanishing breed. Climate change is hammering Adélie penguins on Antarctica's Torgersen Island, Jennifer Blum says.

atmosphere is connected to the ocean, and how they impact the ice in a beautiful chain of events.”

“People used to think Antarctica responds to global warming like a giant ice cube”—with rough uniformity—says Amelia Shevenell, a paleoclimatologist at the University of South Florida, St. Petersburg. “We now know this is not the case.”

Ghosts in the machine

Antarctica's climate is strongly affected by westerly winds that buffet the Southern Ocean. High atmospheric pressure over the midlatitudes pushes air toward the poles. As the winds rush south, they turn eastward with Earth's rotation. In the past few decades, air pressure along the Antarctic coast has often been lower than in previous decades, which strengthens the westerlies and drives them farther south, bringing more warm air to the Antarctic Peninsula. “In years when this happens, there is a 70% chance that the peninsula is warmer and has less sea ice,” says Sharon Stammerjohn, an oceanographer at the University of Colorado, Boulder.

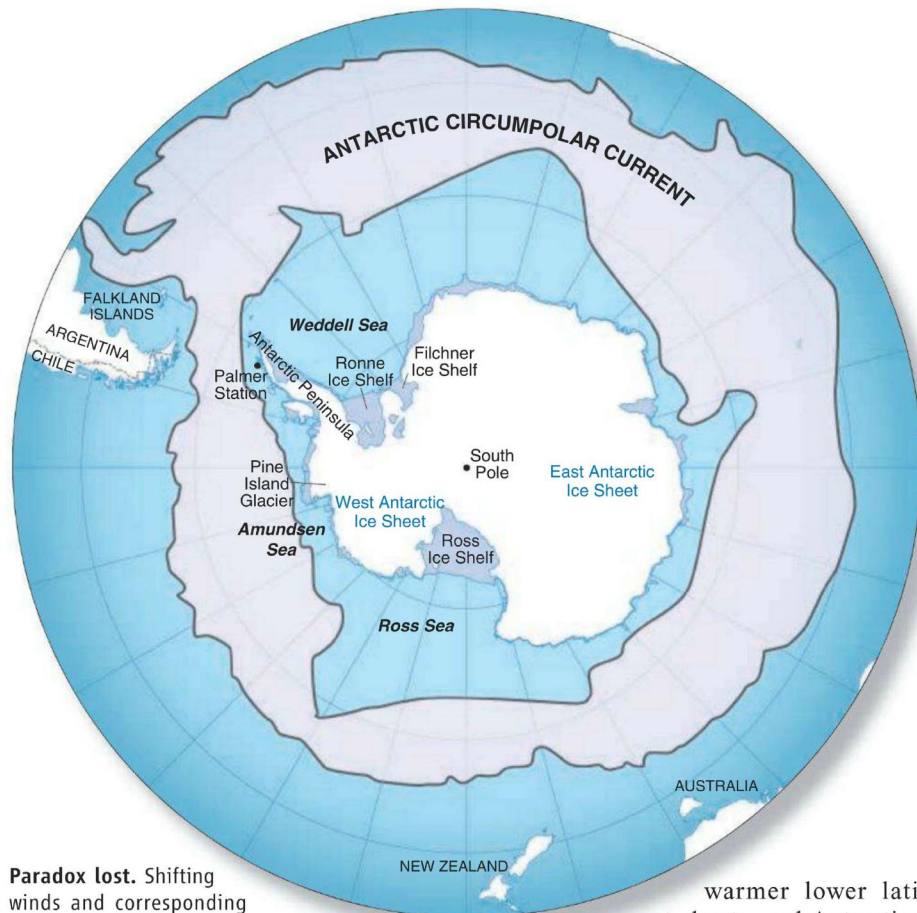
Human activity is partly to blame. Thompson's group, working with climate scientists Susan Solomon of the U.S. National Oceanic and Atmospheric Administration and Nathan Gillett of the University of Victoria in Canada, has found that changes in westerlies during austral summer are caused

by the ozone hole over Antarctica, a thinning of the ozone layer in the stratosphere due to decades of consumer use of halogenated chemicals. Ozone absorbs solar radiation; the cooling strengthens surface westerlies and nudges them toward the South Pole. Climate models show that rising concentrations of greenhouse gases amplify that effect on the westerlies. At the same time, lower coastal air pressures suck cold winds from the pole, spreading a deep chill across the Antarctic interior. This helps explain the apparent paradox of the peninsula warming even as average temperatures in most other parts of the continent—except the Amundsen Sea in western Antarctica—have budged little.

Ocean temperatures are another ghost in the machine that could explain what's happening in the Amundsen region, which has experienced accelerating ice loss in recent decades. Sea surface temperatures in the central tropical Pacific, near the International Date Line, have been rising since the early 20th century. The warmer water heats the overlying air, which rises, expands, and meanders south, getting a push from the subtropical jet stream off eastern Australia. About 50° south of the equator, a high-pressure center steers the air mass in the same direction as the westerlies.

"You can think of the westerlies like a tightly wound guitar string," says David Battisti, a climate scientist at the University of Washington, Seattle. "Pinging on that guitar string by changing tropical convection, you send out waves." His team's modeling shows that as westerlies intensify, loops of warm air detach and pile up over the Amundsen Sea. Cyclones steer this warm air into the continent's interior. That could explain the correlation between warming in the central tropical Pacific and rising winter temperatures in western Antarctica, Battisti says.

The El Niño–Southern Oscillation (ENSO) is plucking a different section of the guitar string. ENSO is a climate pattern that lurches between two states: El Niño, when tropical eastern Pacific surface waters are unusually warm, and La Niña, when they are exceptionally cool. By analyzing all ENSO events in the past 30 years, Stammerjohn and colleagues found that El Niño is closely linked to warming and light sea ice in the Amundsen Sea, but cooling and heavy sea ice in the Antarctic Peninsula. Modeling shows that El Niño tends to induce a persistent high pressure center west of the peninsula. This draws cold winds from the continental interior to the peninsula and pushes warm winds from the ocean over the Amundsen Sea.



Paradox lost. Shifting winds and corresponding ocean changes explain a seeming contradiction: the Antarctic Peninsula and Amundsen Sea growing warmer even as the continent's interior keeps its cool.



Nearing the tipping point?

It's not just the air that's growing warmer. The westerlies over the Southern Ocean produce the strongest ocean current on the planet, the Antarctic Circumpolar Current. As much as 4 kilometers deep and 1000 kilometers wide, the current moves 140 million tons of water per second. "Any changes in this powerful current will have a profound impact," says Michael Meredith, an oceanographer at the British Antarctic Survey in Cambridge, U.K.

As the westerlies are propelled farther south by the ozone hole and atmospheric warming, they tug the Antarctic Circumpolar Current south, pushing ocean heat from

warmer lower latitudes toward Antarctica.

"The stronger winds also stir up more eddies," Meredith says. "These swirls of ocean currents, tens of kilometers in diameter, are effective engines to pump warm water towards Antarctica." The Southern Ocean is already feeling the heat. Since the 1950s, the Antarctic Circumpolar Current's sweet spot—its warmest water at 700 to 1000 meters below the surface—has increased by about 0.2°C, twice as much as the global average warming at that ocean depth. Through research cruises and year-round monitoring at Palmer Station, Stammerjohn and colleagues have charted a steady rise in ocean

heat content since the 1990s in waters along the peninsula's continental shelf. They estimate that 80% of the added warmth is from a greater volume of deep, warm water welling up onto the shelf.

This kind of upwelling is unique to the Southern Ocean. As the westerlies drag the currents around Antarctica, surface water flows north with Earth's rotation, and is replaced by warm deep water from below. "At the peninsula, the circumpolar current skirts right along the coast, and stronger winds could easily lift up more deep water onto the shelf," says Ducklow, an ecologist at the Marine Biological Laboratory in Woods Hole, Massachusetts. On

the continent, meanwhile, climate models forecast that cyclones caused by central Pacific warming could also flush more warm deep water onto the continental shelf in the Amundsen Sea. There, a robotic submarine has probed beneath Pine Island Glacier, which is sliding into the ocean at a rate of 4 kilometers a year. The sub's missions are shedding light on how deep water may erode ice sheet stability (see sidebar).

The climate changes down south could have far-reaching ramifications. The pre-eminent concern is sea levels: how high they will rise as ice sheets succumb to a global warm-up. A less intuitive consequence is how changing conditions in waters off Antarctica will affect the global ocean's cooling and gas exchange. Climate change could influence the amount of cold salty water that sinks to the ocean bottom. Antarctic Bottom Water, as it's called, pools at only a few spots around the continent, where seawater is cooled by overlying air and rendered saltier as ice forms. More than 60% is concentrated in one location: the Weddell Sea, east of the Antarctic Peninsula, where projected changes in ocean currents could accelerate ice sheet disintegration, making the surface water fresher and lighter.

In recent decades, a combination of warmer air, lighter sea ice, and more ice shelf melting has made waters fresher and lighter in parts of the Antarctic coast, says Gregory Johnson, an oceanographer at the University of Washington, Seattle. From ship-based surveys in the Southern Ocean, Johnson and colleagues found that the volume of Antarctic bottom water has decreased by 10% per decade over the past 30 years. Bottom water circulates globally and mixes with warmer waters above, much like refrigerants cooling a fridge. Less bottom water, Johnson says, "could affect heat exchange and climate regulation on a global scale." Loosening that bridle on ocean temperatures could have catastrophic consequences for the global climate and for sea life, experts say.

In the meantime, there are fears that the Southern Ocean may be losing capacity to store carbon in its depths, which may spur global warming. It accounts for about 40% of the total global ocean uptake of atmospheric carbon dioxide. Stronger upwelling stirs up deep carbon-laden waters. "This can increase carbonate levels in the surface and lead to more outgassing and less uptake," says Corinne Le Quéré, a biogeochemist at the University of East Anglia in Norwich, U.K. Her team has found that the Southern Ocean has absorbed less carbon in the past

Slip Sliding Away

Pine Island Glacier is part of the weak underbelly of the West Antarctic Ice Sheet. It and other glaciers that flow into the Amundsen Sea are at the foot of the fastest melting ice shelves on the continent. Pine Island Glacier is sliding into the ocean at a rate of 4 kilometers a year and contributes a whopping 4% to the recent global sea level rise of 3 millimeters a year.

To find out what's going on, in 2009 a team led by oceanographer Stanley Jacobs of Columbia University's Lamont-Doherty Earth Observatory in Palisades, New York, and glaciologist Adrian Jenkins of the British Antarctic Survey sent a submarine beneath the Pine Island Glacier Ice Shelf: the floating tongue of ice where land-bound glaciers meet the ocean. Compared with data gathered 15 years ago, their findings were alarming. They detected 50% more meltwater gushing from the cavity and a widening gap between the ice and an underwater ridge crest, probably due to sustained melting.

As a result, the current flowing into the ice cavity was 77% stronger in 2009 than it was in 1994. "What goes into the cavity is the warm deep water," Jacobs says. "You can trace it all the way to the edge of the continental shelf hundreds of kilometers in the north." The deep water is 3.5°C warmer than the freezing point at 1 kilometer below the ocean surface, where it encounters the ice. "It's very effective at melting ice from the shelf base at that depth," Jacobs says. As the shelf thins, the ice loses its grip on the sea floor and ice moves more rapidly into the sea.

This increased basal melting has turned out to be a common mechanism of ice loss in coastal Antarctica. In a recent survey, glaciologist Hamish Pritchard of the British Antarctic Survey and his colleagues found that basal melting accounts for thinning at 20 out of 54 ice shelves. "The fastest thinning takes place on the coast of western Antarctica," Pritchard says. "It's invariably at places where deep warm water can access ice shelves through submarine trenches across the continental shelf."

Other regions also may not be safe from melting for long, says Hartmut Hellmer, an oceanographer at the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven, Germany. His group's climate models found that water in the cavity underneath the Filchner-Ronne Ice Shelf in the Weddell Sea would be 2°C warmer by the end of the 21st century under a conservative estimate of carbon emissions and economic growth. Warmer cavity water would increase basal melting 20-fold, resulting in 1.6 trillion tons of ice loss a year. That would freshen waters at the ocean surface and allow deep warm water of the circumpolar current, which now largely stays away from the continental slope, to penetrate into the trough underneath Filchner-Ronne, an ice shelf the size of Spain that's fed by ice streams from both the West and East Antarctic ice sheets. That could conjure a nightmare scenario: "There would be serious implications for the stability of both ice sheets," Hellmer says.

—J.Q.



Bombs away. A crack in Pine Island Glacier, imaged in late 2011, presages the calving of a 900-square-kilometer iceberg.

30 years despite rising industrial emissions.

All climate signals point to a grim outlook for the frozen continent. Models predict that the westerlies will grow stronger and drift southward; the central tropical Pacific will continue to warm; areas that are not warming now, such as the Weddell Sea and the Ross Sea, will become hot spots in less than a century if greenhouse emissions are not curbed; and ENSO cycles will grow more intense and flip more frequently. "The implications for Antarctica will be dire," Shevenell says. Consequences could include widespread ice sheet disintegration,

ecosystem deterioration, and disruption of global ocean circulation.

The Antarctic Peninsula is already at the vanguard of climate change—and may be approaching the point of no return. "It might be a good place to develop a capability to predict when the Earth system is close to the tipping point," Ducklow says. "It's like the canary in a coal mine for what's coming for the rest of the planet."

—JANE QIU

Jane Qiu is a writer in Beijing. Her trip to the Palmer Station was supported by the Marine Biological Laboratory's Logan Science Journalism Fellowship.



LETTERS

edited by Jennifer Sills

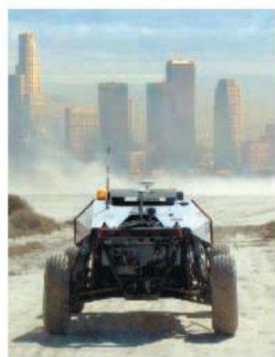
Small Science: Radical Innovation

B. ALBERTS' RECENT EDITORIAL, "THE END OF 'SMALL SCIENCE'?" (28 September, p. 1583), resonated with me. I think many of his conclusions apply not only to biology, but to other fields of science and engineering.

One example from the late 1960s was Multics, a joint effort of MIT and Bell Labs to create a new generation of operating systems (*1*). This was originally planned as a coordinated effort for dozens of researchers collaborating on what was then a big computer. It was preempted

by an offshoot called Unix, a project on which nearly all of Multics' goals were achieved much more quickly by a single pair of researchers on a much smaller minicomputer. Unix soon became widely recognized as a big success; its present-day descendants include Linux. Multics eventually stumbled under its own weight and died.

Another example is the development of fully autonomous ground vehicles and human-sized robots. Starting in 2005, the Defense Advanced Research Projects Agency (DARPA) began offer-



Grand Challenge course.

ing "Grand Challenge" prizes for the development of fully autonomous ground vehicles capable of completing tasks formerly achieved only by vehicles with human drivers (*2*). In 2005 to 2006, the goal was to drive across a challenging 150-mile off-road course in the Mohave Desert from Barstow, California, to Primm, Nevada. In 2007, the goal was extended to drive autonomously in a mock urban environment including heavy traffic. All totaled, these competitions have attracted over 100 competing teams, each of which typically consists of only about a dozen individuals. This research and development strategy has arguably produced better results faster and with much less expense than the more traditional big-scale strategy of deploying hundreds of people in a single focused effort managed through a standard organization chart.

Of course, there are situations for which the more traditional management strategy may fare better. But when the primary goals are new understanding or radical innovation, rather than incremental improvements, smaller is often better than bigger. In more abstract theoretical subjects such as mathematics, most important research publications have only a single author, and papers with more than three coauthors are extremely rare.

ELWYN BERLEKAMP

Professor Emeritus, University of California at Berkeley, Berkeley, CA 94720, USA. E-mail: berlek@gmail.com

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Small Science: View from Developing Nations

IN THE EDITORIAL "THE END OF 'SMALL SCIENCE'?" (28 September, p. 1583), B. Alberts asks whether the era of "small-science" projects is coming to an end. He hopes not. I share this sentiment, from the perspective of developing countries where science budgets are small.

It seems clear, however, that big, headline-grabbing projects will likely continue to increase. Thus, the leaders of these projects should seize the opportunity to enable and inspire the next generation of scientists in developing countries. Developing countries do not have the budgets to initiate such "big-science" projects, but they do have ample talent to contribute. Unfortunately, ENCODE—

just like the Human Genome Project and others—included scientists from only one developing nation (China).

An exceptional example of integrating developing countries into big-science projects is the decision to award the Square Kilometre Array radio telescope to Africa (*1*). The ripple effects in the media, government, and rest of society are noticeable in South Africa and promising for public support for science. Such support is critical if we are to bridge the gap between science in developed and developing countries, in order to address inequality and the interconnected sustainability problems facing the world.

The scientific community often points a finger at the failure of governments to address these issues. The scientific community, how-

ever, also needs to take care of its responsibility and opportunities to help bridge that divide. Big-science projects have the power to make a substantial difference in this regard.

BERNARD SLIPPERS

Department of Genetics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, 0002, South Africa. E-mail: bernard.slippers@fabi.up.ac.za

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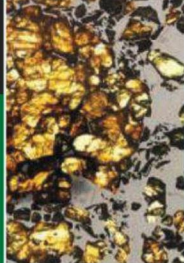
Small Science: Big Science Will Prevail

IN CANADA, I HAVE ALSO RECOGNIZED THE escalation of big science (not only ENCODE) as an increasingly dominant aspect of modern science culture, perhaps indirectly cata-



Island archaeology
revisited

895



Pallasite origins

897

lyzed by the Manhattan project as well as the Human Genome project ("The end of 'small science'?", B. Albert, Editorial, 28 September, p. 1583).

I attribute the situation to our academic culture. We encourage growth and renewal, but impose no limits (except money) on the size of the enterprise devoted to a specific project.

In my view, big science will prevail. The positions big science makes available provide a safety net for the plethora of well-trained Ph.D.'s, who are finding careers in academia increasingly rare, and big-science projects and successes are much more visible to politicians and the many others who are ignorant of science culture.

LOU SIMINOVITCH

Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, M5G 1X5, Canada. E-mail: lsiminovitch@mtsina.on.ca

Small Science: High Stakes

IN HIS EDITORIAL "THE END OF 'SMALL science'?" (28 September, p. 1583), B. Alberts perceptively highlights the threat of erosion in support for small, fundamental investigations on which a deeper understanding of the complexity of biological phenomena rests. There are other potentially insidious dangers.

The displacement of small science is also likely to have repercussions on science pedagogy. In biomedical fields, small science has historically provided the training ground on which budding scientists develop the technical and creative mastery of their craft. The curtailment of small science thus calls for a critical reevaluation of, and perhaps a transformative new approach to, the biomedical curriculum. Aside from the impact on the training of future investigators, to what extent big science may be integrated into a liberal arts curriculum (and the consequences for a scientifically literate citizenry) (1) requires thoughtful collective consideration.

The primacy of big science creates incentive structures that may further impinge on the work of innovative small science. These incentive structures may be reproduced in, or refracted through, funding bodies, academic promotion committees, and journal editorial

boards. The diversity of scale in research may thus come to be under siege from all quarters.

Finally, big science and small science have different practices, norms, and infrastructures (2). Does big science facilitate successful collaborative work at the expense of substantially reduced heterogeneity in research approaches? Does big science guide scientific advance toward its own perpetuation and away from certain lines of inquiry? No less than a vision for the future of biomedical research is at stake.

ERIC R. GAMAZON

Section of Genetic Medicine, Department of Medicine, University of Chicago, Chicago, IL 60637, USA. E-mail: egamazon@medicine.bsd.uchicago.edu

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Disease Prevention: Experiments in Nature

THE SPECIAL SECTION ON DISEASE PREVENTION (21 September, p. 1466) reminded me of the late E. L. Wynder's key role in the development of this field. Wynder developed the idea of using "experiments in nature" as clues to the role of the environment in understanding disease causation and prevention. For example, he and his coauthors pointed to the fact that gastric cancer, a common cancer in the United States at the turn of the century, was relatively rare during the latter half of the century. They attributed the decline not to medical intervention but to the introduction of refrigeration and the ensuing decline in the use of nitrates for food preservation. Bolstering this view was the finding that nitrates are readily converted into carcinogenic nitrosamines in the acidic environment of the stomach (1). Wynder thus provided an interesting example of how a disease could be suppressed (but not cured) by a technological innovation rather than a medical intervention.

L. A. COHEN

Nutrition and Cancer: An International Journal, Northampton, MA 01060, USA. E-mail: leonardcohen65@verizon.net

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Disease Prevention: Vitamin D Trials

ALTHOUGH THERE IS A DEBATE ON CUT-OFFS for appropriate vitamin D supplementation ("Uncertain verdict as vitamin D goes on trial," K. Kupferschmidt, News, special section on Disease Prevention, 21 September, p. 1476), clinicians universally agree that vitamin D deficiency is detrimental for bone health (1). We also know that vitamin D overdosing can be toxic. What quantity will prevent both deficiency and toxicity?

To find the ultimate vitamin D dose and to evaluate its effectiveness, researchers should learn from randomized controlled trials (RCTs) of drugs for diabetic or hypertensive patients, who were usually treated with the goal of achieving well-defined targets, such as certain HbA1c or blood pressure levels. In the case of vitamin D, this would mean performing RCTs in individuals with overt vitamin D deficiency and using doses to achieve optimal vitamin D levels.

Instead of performing these kinds of RCTs, the design of the ongoing vitamin D trials resembles previous (disappointing) vitamin trials, which attempted to establish a dose that should fit for the entire population (2). If the current vitamin D trials fail, we will ask ourselves why we did not perform RCTs exclusively in vitamin D-deficient patients rather than attempting to base conclusions on a heterogeneous population. Subgroup analyses of existing trials will not satisfy health authorities.

STEFAN PILZ,^{1,2*} FEMKE RUTTERS,¹
JACQUELINE M. DEKKER¹

¹Department of Epidemiology and Biostatistics, EMGO Institute for Health and Care Research, VU University Medical Centre, 1081 BT, Amsterdam, Netherlands. ²Department of Internal Medicine, Division of Endocrinology and Metabolism, Medical University of Graz, 8036, Graz, Austria.

*To whom correspondence should be addressed. E-mail: s.pilz@vumc.nl

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Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

WOMEN IN SCIENCE

A Classic Continued Up to the Present

Georgina M. Montgomery

As the old adage goes, well-behaved women don't make history. This saying certainly holds true for the small number of "clever, astute, hardworking, and determined" women who harnessed their individual and collective frustration about pervasive prejudice in the sciences to demand recruitment, recognition, representation, and promotion of women scientists. In *Women Scientists in America: Forging a New World Since 1972*, Margaret Rossiter guides us from the "rather quiet, mundane, even ladylike" emergence of female researchers' first interest groups to the direct confrontations they would take on in pursuit of equity in the sciences.

Rossiter (a historian of science at Cornell University) introduces dozens of women whose activism evokes a sense of admiration. Mary Gray, a founding member of the Association for Women in Mathematics, merged her skills as professor, lawyer, and feminist in her expert testimony for Senator Edward Kennedy's 1979–1980 "women in science" bill, which established an advisory committee on women and minorities for the National Science Foundation. Janet Welsh Brown, leader in the Federation of Organizations for Professional Women, consolidated hundreds of women's voices into one too loud to ignore even as she dedicated herself to minority and disability issues. Working at the American Association for the Advancement of Science, she produced *The Double Bind: The Price of Being a Minority Woman in Science* (1) and a booklet, *Barrier-Free Meetings* (2).

Rossiter sketches the biographies of women scientists who fought prejudice in the sciences, and particularly in universities, through lawsuits made possible in the United States by the Equal Employment Opportunity Act of 1972. Specific cases of discrimination combined with status-of-women reports by professional associations and universities demonstrated widespread inequality in the

hiring and promoting of women and minorities. Individuals such as Sharon L. Johnson, a biochemist at the University of Pittsburgh Medical School, and Shyamala Rajender, a chemist and instructor at the University of Minnesota, took on their respective institutions in the courts. Such actions were important as these women fought "for themselves, for their 'class' of fellow female academics ... for colleagues in their fields across the nation, and for future women scientists." All too often their own careers were derailed even as they established opportunities for others.

At the same time, multiple local programs began to warm the "chilly climate" that had greeted older women scientists at the start of their careers and had far

too frequently persisted well beyond tenure. Between 1970 and 1980, the annual number of U.S. female undergraduates attaining bachelors' degrees in the sciences increased from 80,000 to 200,000. Engineering schools hired consultants to create mechanisms for mentoring their growing numbers of female undergraduates, as issues of equity emerged in laboratories and other facilities and led to retention problems. Male-only schools responded by going co-ed. It quickly became apparent that it was essential to create programs to offset circumstances that the first arrivals at Princeton

University, for example, described as lonely and isolating.

In the last decades of the 20th century, as numbers increased in some but not all fields, recruiting became a priority. Powered by "the largely volunteer zeal of thousands of individuals," a range of programs showed girls the new opportunities emerging for women in science, technology, engineering, and mathematics (STEM) fields. The Women and Mathematics initiative, started in 1974, involved college educators visiting secondary schools. A year later, with modest corporate funding thanks to a proposal written by Gray, speakers targeted tenth-grade girls in order to help them acquire the requisite skills for advanced work in science and engineering. Many of the feminist mathematicians became self-appointed "watchdogs" who spoke out when girls' apparent "underperformance" in mathematics was identified as genetic rather than the result of such factors as math anxiety.

During the 1980s, data revealed that the growing numbers of female science undergraduates had not led to proportionate increases in career opportunities in academe or corporations. Looking around at their departments and professional associations, individuals and groups "created a recurring drumbeat, Why so few?" Individuals responded to this sense of isolation by seeking mentors and forming support groups. A group of San Francisco Bay Area women scientists, for example, gathered biweekly to exchange experiences and advice, ultimately sharing their approach to collective mentoring at the 1994 meeting of the American Society for Cell Biology.

As the 20th century drew to a close, the responsibility of making STEM disciplines more inclusive increasingly fell on university administrators. A 1999 Massachusetts Insti-

Women Scientists in America

Volume 3, Forging a New World Since 1972

by Margaret W. Rossiter

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Shown in different lights. Two portrayals of neuropathologist Margaret Zee Jones: Posed with goats at the Michigan State University Veterinary Research Farms (1986, left) and examining brain slices for abnormalities (1994, right).

The reviewer is at Lyman Briggs College and the Department of History, Michigan State University, East Lansing, MI 48825, USA. E-mail: montg165@msu.edu

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tute of Technology report (3) revealed that tenured women scientists had “been treated unequally for years. ... At the peak of their careers they were isolated, powerless, and almost invisible.” In response, President Bill Clinton invited Nancy Hopkins (a molecular biologist at MIT who led the report) to speak at the 1999 celebration of Equal Pay Day. Hopkins soon was touring the country, electrifying fellow female scientists and opening the eyes of some skeptical males.

Like Hopkins’s talks, *Women Scientists in America: Forging a New World Since 1972* may send readers on an emotional journey. Rossiter’s extraordinarily detailed account offers compelling data alongside the multiple stories of individual women, both those thwarted by discrimination and those who emerged as outstanding success stories. The inequities are all too evident and persistent, while the strategies—mentorship, outreach, and challenging the status quo—remain those of activist men and women still involved in promoting women and minorities in science. This book should be read by skeptics who don’t believe that there is persisting prejudice. It also provides inspiration and ideas for those who relish the stories of women who now deservedly do make history.

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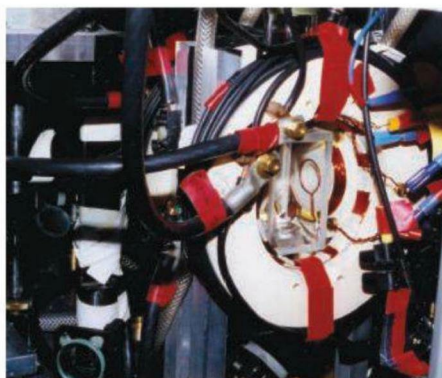
HISTORY AND PHILOSOPHY OF SCIENCE

A Revolution of Its Own

Daryn Lehoux¹ and Jay Foster²

No one can deny that science has been successful, but why has science been so successful? Surely it’s not an accident, not just a long run of good luck. The reason why, or so most of us learn from our science classes and textbooks, is that the sciences have a distinctive method. In *The Structure of Scientific Revolutions* (1),

¹Department of Classics, Queen’s University, Kingston, ON K7L 3N6, Canada. E-mail: lehoux@queensu.ca. ²Department of Philosophy, Memorial University of Newfoundland, St. John’s, NF A1C 5S7, Canada. E-mail: ajfoster@mun.ca



The patchiness of scientific practice. Apparatus for producing Bose-Einstein condensate.

Thomas Kuhn argued that the sciences do not share anything quite so rigorous as a method. The success of science, he argued, is a consequence of a more general structure of scientific inquiry.

Since *Structure* was first published in 1962, it has sold over one million copies and has been translated into 16 languages. This book is the rarest of things, an academic bestseller. Pick any list of the top 100 most influential books of the 20th century—or even the most influential books of all time—and it’s very probable that *Structure* will appear on that list. Philosopher of science Ian Hacking, who introduces this 50th-anniversary edition of the book, tells us: “Great books are rare. This is one. Read it and you will see.” He is right on the mark.

What made Kuhn’s view of science so different and so attractive? Before Kuhn, philosophers had been focused on trying to state what the scientific method should be. But he suggested that we should pay attention to how science is done—how scientists actually work—rather than to how philosophers think it ought to work. If you want to know how science is carried out, then in one way or another, you are going to have to look at the history of science. It’s this history that shows us how the sciences work in action.

The opening line of *Structure* is thus a powerfully subversive statement: “History, if viewed as a repository for more than anecdote or chronology, could produce a decisive transformation in the image of science by which we are now possessed.” If you want to understand the sciences, says Kuhn, examine how scientists work and how their work has changed over time. Over the past half-century, this approach to studying the sci-

ences has given us a radically different perspective on scientific practice and progress.

Most accounts of scientific method describe it as being empirical and logical. Past this very superficial consensus, there is often little agreement. Different scientists working in different scientific disciplines will likely disagree about anything more specific. To answer more specific questions, many still lean heavily on the ideas of Karl Popper (who, Hacking observes, was “[b]efore Kuhn ... the most influential philosopher of science—... the most widely read, and to some extent believed, by practicing scientists”). Popper’s familiar view, very roughly, is that scientists should formulate empirically falsifiable hypotheses, devise empirical tests for the hypotheses, and then conduct these tests (2). If, in any instance, the empirical test fails, then logic demands that the hypothesis must be rejected. This method, usually simply called falsification, has its roots in deductive logic, specifically the *modus tollens* (literally, “the mode that denies by destroying”).

Despite all of its merits, the Popperian method isn’t entirely satisfying. It leaves even the best scientific hypotheses with the status of being not so much true as “not yet falsified” or, at the very best, “approximately true.” Even Popper’s famous detractor, Carl Hempel, agreed that science did not attain truth but only “high probability” (3). Surely our most-established scientific ideas—for example, that a hydrogen atom has one electron and one proton—are something more than approxi-

mate truths, something more than just highly probable? Kuhn would have agreed. He thought that no single method could successfully describe all work in the sciences. The alternative to understanding the sciences as having a methodological unity is to understand them as a general mode of inquiry that is oriented toward working out specific problems or “puzzles.”

Scientific puzzle-solving is guided or framed by specific attention-grabbing successes. These “paradigms” or “exemplars” structure inquiry by providing a touchstone for the coordination of research, the formulation of experimental routines, and, in many cases, a theory worthy of further articulation. Right now, post-Watson-and-Crick genomics and high-energy physics are the most publicly visible paradigms. Newton’s *Principia Mathematica* is a classic historical example of such a success. The basic tools and ideas

The Structure of Scientific Revolutions

Fourth Edition

by Thomas S. Kuhn,
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Ian Hacking

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provided in the *Principia* were worked out by Laplace, Lagrange, Maupertuis, Cavendish, Gauss, and many others. Gravitational attraction was reexpressed and variously articulated in ways Newton could not have anticipated and might not have endorsed.

The interesting feature of paradigms is that they aren't "discoveries" in the traditional sense. They aren't worked-out chunks of knowledge. They need not, at their inception, be approximately true or even highly probable. Instead, the most important characteristic of a paradigm is its fruitfulness. The active working-out of a paradigm Kuhn called "normal science." As normal science develops, he argued, a number of exceptions or difficulties emerge. By the falsificationist account, the emergence of an exception or anomaly—any anomaly—demands the rejection and subsequent revision of theory. Kuhn disagreed: "No process yet disclosed by the historical study of scientific development at all resembles the methodological stereotype of falsification by direct comparison with nature."

So long as a paradigm continues to be useful for the elaboration of theory and practice, anomalies are simply accumulated, tolerated, or explained away (as minor exceptions, experimental errors, or being only marginally relevant). Research groups almost never give up on a research program simply because it has anomalies. Those become relevant only when a paradigm begins to exhaust its productivity, or when a researcher or research team begins to worry enough about some anomalies so as to question the established paradigm.

When this happens, Kuhn thinks, a science becomes ripe for a distinct rupture—a scientific revolution. But, importantly, this revolution is not just an out-and-out rejection of the old paradigm; it's not the logical falsification of a theory in light of incontrovertible evidence, as Popper would have us see it. In the midst of a scientific revolution, there are likely to be a number of competing candidates for the research agenda. Advocates of a new paradigm vie with advocates of the old. Should the old paradigm be patched up? Or should there be a whole-cloth replacement with a new paradigm? Kuhn argued that these questions are not answered by straightforward appeals to reason and evidence.

In the midst of a revolution, what count as good evidence and reason in a discipline are themselves open to dispute. In the Chemical Revolution of the 18th century, Lavoisier advocated a blanket replacement of the old phlogiston theory and argued for the centrality of the mass balance to chemical analysis. Phlogiston chemists maintained that prob-

lems with their work could be fixed. Some claimed that mass was properly a physical property and so not part of chemistry. The Chemical Revolution lasted 50 years or so. At its end, something like Lavoisier's chemistry "won." However, Kuhn argues, that victory was achieved not by the tabling of incontrovertible evidence one way or the others—no such evidence was available. Rather, the relevant communities of practicing chemists gradually found Lavoisier's approach more fruitful.

Kuhn's idea of scientific revolution—which has given its name to any number of undergraduate courses in the history of science—raises an important and interesting question: what is the status of the new, post-revolutionary paradigm with respect to the old? Kuhn's answer is that the new and old paradigms are simply "incommensurable." The incommensurability thesis, Kuhn's most controversial idea, maintains that when scientists change paradigms through a scientific revolution, important terms or concepts change their meaning or even cease to be meaningful in the new paradigm.

Let's take a concrete example. When Copernicus proposed replacing Ptolemy's geocentric cosmology with a heliocentric one, clearly the meaning of the word "planet" had to change (the objects that it covered now included Earth). Even more important, if Copernicus's view were to be accepted, it wasn't just the definition of planet that would have to be rethought. Researchers were left with a much larger explanatory vacuum. Previously acceptable explanations of how and why things moved—a whole physics—would also need to be changed. If Earth is in fact moving around the Sun, then why do heavy things tend to fall to the ground on Earth? The old conflux of geocentrism, the "natural motion" of heavy bodies toward the center of the cosmos (i.e., Earth) and the necessary motion of planets-made-of-aether around that center, could find no equivalent in the new paradigm.

Put simply, the terms of the new way of working are not translatable into those of the old way. The inability to straightforwardly translate between competing paradigms without interpretation is their "incommensurability." The tree of knowledge grows, says Kuhn, but it is also pruned. Philosophers, perhaps understandably, treated this notion with alarm, incredulity, and sometimes derision. American philosopher and mathematician Hilary Putnam declared it to be "totally incoherent" (4). Nevertheless, it has not proved easy, after Kuhn, to formulate a way of understanding how paradigms

change without invoking incommensurability or something similar (5).

Many philosophers worry that incommensurability leads us straight to an opportunistic relativism in which there are many equally valid and effective ways to describe and work in the world. If we accept Kuhn's argument that science is organized around specific paradigms, and that different paradigms come with different criteria of justification, then what stops someone from claiming that, for instance, creationism (or anything else) is simply a different, incommensurable paradigm? Kuhn would never have endorsed such claims. The error that underlies them is to infer that the rejection of a single scientific method, such as falsification, entails the acceptance of anything whatsoever as being scientifically credible. That is clearly not the case. Saying that there are different ways of doing science is not the same as saying that anything at all qualifies as science.

What, then, rules out, say, creationism as science? Simply this: creationism isn't fruitful; it's dogmatic. It generates no new insights, and it produces no new outcomes, no new ways of generating evidence. Science, in Kuhn's image, must be productive or its paradigm must change.

Few today would call themselves Kuhnians in any pure or committed way. Yet, it is hard to find many areas of the history or philosophy of science that have not been touched by Kuhn's ideas. Philosophers of science are much more attentive to the history of science. They are also much more interested in understanding how the sciences are practiced. They are less concerned with legislating how science should be done. The Kuhnian image of science has reshaped our understanding of the scientific enterprise and human inquiry in general. If you haven't already read *The Structure of Scientific Revolutions*, the publication of this inexpensive 50th-anniversary edition offers a perfect excuse to do so on your next flight.

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SUSTAINABILITY

The Green Economy Post Rio+20

Edward B. Barbier

The distinguishing theme of the June 2012 United Nations (UN) Rio+20 conference was the “green economy” or, more precisely, how economies can achieve “green growth” (1). Although many green economy policies were endorsed in Rio, no new international commitments emerged. I outline steps that could be taken in the aftermath of Rio to salvage the momentum in support of green growth.

According to a key background document for Rio+20 (2), “In a green economy, growth in income and employment should be driven by public and private investments that reduce carbon emissions and pollution, enhance energy and resource efficiency, and prevent the loss of biodiversity and ecosystem services. These investments need to be catalysed and supported by targeted public expenditure, policy reforms and regulation changes.” Several case studies and analyses illustrate how such policies can foster greener growth (2–5).

Transitioning to a green economy requires a mix of short- and long-term policies, a different mix of policies and instruments for rich as opposed to poor countries, and overcoming the political difficulty of implementation (2–5). Some trade-offs involved in adopting selected green growth policies are depicted in the table. The final declaration of the conference endorsed many of these green economy policies but stopped short of any new international commitments, such as phasing out of fossil fuel subsidies, an agreement on managing global oceans, creating a World Environmental Organization, or breaking the deadlock on climate change negotiations (6). Nevertheless, Rio+20 could have made a major contribution to “greening” the world economy if it had addressed three important policy challenges.

Unhelpful, Irrelevant, and Ignored by G20

First, Rio+20 should have declared that it is an anachronism in the 21st century. Holding an international conference every 10 or 20 years to review and promote progress on sustainable development is no longer helpful or relevant. It is unhelpful because the world cannot wait so long to determine whether the current push for “green growth” is suc-

cessful. It is irrelevant because relegating the green economy agenda to international environmental conferences, such as Rio+20, means that economic policy-makers can conveniently ignore green growth as solely a peripheral “environmental” concern.

Adopting the theme of green economy at Rio+20 was laudable. However, this agenda received little support at the concurrent G20 conference held in June in Mexico. The G20 summit, an annual meeting of leaders from the 20 largest and most populous economies in the world, is a forum for discussing and coordinating global economic policy (7). The focus of the G20 summit was on the current global economic crisis, and green economy issues did not receive strong endorsement as part of the policy solution to the crisis. The Mexico G20 Leader’s Declaration did state that “We commit to maintaining a focus on inclusive green growth as part of our G20 agenda and in the light of agreements reached at Rio+20 and the United Nations Framework Convention on Climate Change (UNFCCC)” (8). However, no concrete actions or policies for achieving “inclusive green growth” were offered by the G20.

In its final outcome document, *The Future We Want* (6), Rio+20 should have challenged the G20 to live up to its pronouncements on “inclusive green growth” through endorsing and adopting concrete green-economy

Sustainable development, a focus at the June United Nations meeting, requires critical endorsement and action by the G20.

policy actions at future G20 summits. Although the G20 is not a global policy-making body, its 20 economies comprise nearly 80% of the world’s population and 90% of global gross domestic product (GDP) (9). By implementing green growth policies, the G20 would not only demonstrate to the rest of the world that global economic and environmental issues are inseparable but also could have a measurable impact on greening the world economy.

Second, Rio+20 and similar environmental conferences often propose targets for action and promise financial commitments without considering the mechanisms by which those funds will be raised. Yet, there is a wide range of global funding sources for environment and development initiatives, including financial transaction taxes; international financing facilities; sovereign wealth funds; and taxes on global arms, tobacco, and the fuel trade (10). Although Rio+20 recommended sustainability reporting for capital markets and abolishing fossil fuel subsidies (6), these are only modest financial proposals. Even support for eliminating fossil fuel subsidies was lukewarm at best; whereas some countries used Rio+20 “to reaffirm the commitments they have made to phase out harmful and inefficient fossil fuel subsidies,” the conference simply urged others to “consider rationalizing inefficient fossil fuel sub-

Local and immediate benefits versus global and long-term benefits				
		Fewer trade-offs	Some trade-offs	More trade-offs
Political difficulty of implementation	Easy	Energy conservation	Improved drinking water and sanitation	Carbon sequestration projects
		Land-use planning	Development of fuel-efficient vehicles	
	Moderate	Public urban transportation	Low-cost clean energy supply	Ocean conservation and fisheries management
			Removal of fossil fuel subsidies	International payment for ecosystem services
			Subsidies for clean energy R&D	Large-scale water management projects
	Hard	Pollution regulation and pricing	Natural resource management and pricing	Global carbon tax
			Sustainable intensification of agriculture	High-cost clean energy supply
			Water pricing	Removal of agricultural subsidies
			Removal of water subsidies	
			Carbon pricing	

Schematic ranking of select green growth policies. The ranking reflects the political difficulty of implementation and trade-offs between local and immediate versus more global and long-term benefits. [Based on (2–5)]

sidies...with the aim of minimizing the possible adverse impacts on their development and in a manner that protects the poor and the affected communities” (6).

Finally, global sustainable development issues need a permanent international champion. The “zero draft” outcome document prepared for Rio+20 proposed that the UN Environment Programme (UNEP) be transformed into a full-fledged specialized UN agency capable of generating binding negotiated agreements and principles among members at its annual general assembly and other official meetings (1). Such status is currently accorded other UN agencies, such as the International Labor Organization (ILO) and the World Health Organization (WHO). Although UNEP should have the same policy status and mandate on behalf of the global environment, Rio+20 failed to endorse this proposal (6).

If these three actions had been enacted at Rio+20, it would have signaled that the “green economy” is not just a current “buzz concept” but represents a profound change in how global economic and environmental issues are viewed by the world community.

The Way Forward

Although an important opportunity for a paradigm shift in international policy was lost at the conference, several steps can still be taken. First, UNEP should be converted into a specialized UN agency for the environment, with a revised and strengthened mandate; stable, adequate, and predictable financing; and equal operating status to other UN specialized agencies. UNEP’s Green Economy Initiative (GEI) influenced significantly the Rio+20 agenda (2) and, along with other international institutions, has created a global research network addressing major knowledge gaps in green growth theory and practice (11). In addition, the GEI aids governments in developing green growth policy strategies, including reconciling competing policy trade-offs (12) (see the table). The UN Economic and Social Council has the mandate to convert UNEP into a specialized agency, a move already supported by African and European nations. If transformed into a specialized agency and adequately funded, UNEP could expand its policy advice, technical assistance, and applied capacity in support of national and regional initiatives on the green economy (13). A specialized UN agency for the environment would significantly improve adoption of green economy policies. However, enhanced global environmental governance will also require greater coordination between the new agency and the UN General Assembly, the UN Economic and Social Council,

and more specialized international environmental agencies such as the UNFCCC.

Second, the G20 should honor its promises from past summits to “make the transition towards clean, innovative, resource efficient, low carbon technologies and infrastructure...and work together on further measures to build sustainable economies” (14, 15). As noted above, the 2012 Mexico summit also pledged “a focus on inclusive green growth as part of our G20 agenda” (8). It is therefore incumbent upon the G20 to develop specific green growth strategies to fulfill such promises. For upcoming summits, the G20 should start discussing how green economy policies and trade-offs could be prioritized for different member countries. Supporters of green growth among the G20—such as Australia, Denmark, the European Union, France, Mexico, South Korea, and the United Kingdom—should collaborate to promote this initiative, which has been endorsed by the G20 Development Working Group.

Finally, the international community must establish urgently new financial mechanisms for long-term funding of sustainable development (10). Such an initiative has precedents. In the aftermath of the 2008–2009 financial crisis, the UN General Assembly established a commission, chaired by Nobel laureate economist Joseph Stiglitz, to examine reforms of the global financial and monetary system (16). The Gates Foundation submitted a report on innovative financing mechanisms for development to the 2011 Cannes G20 Summit, which estimated that an additional \$85 billion in assistance annually could be raised through a modest combination of fuel and tobacco taxes, financial transaction taxes, and investments from dedicated bond and sovereign wealth funds (17). Employing a full range of such innovative financial mechanisms could boost annual funds by as much as \$1.1 trillion (10). The international community must consider such proposals to be an important component in the process of promoting the transition to a green economy. Even in conventional development assistance, the shortfall between aid needs and commitments is growing. For example, the cost of funding the UN Millennium Development Goals of reducing global poverty is estimated to require additional development assistance of \$83 billion annually, but over the past 10 years, developing countries have received only \$37 billion in total extra aid for this purpose (18). In October 2012, the European Commission backed plans by 10 countries to impose a financial transaction tax, which should demonstrate the viability of such alternative financing instruments to the rest of the world.

The green economy paradigm starts with the premise that the separation of economic development and environmental policies is artificial. Although Rio+20 may have missed the opportunity of delivering any specific policy agreements for achieving green growth, the conference has at least challenged the world community to take this theme seriously. In the aftermath of Rio+20, the international community must take urgent and concrete steps to break down false barriers separating environment and development concerns. The world cannot afford to wait another 20 years to address such issues again.

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CELL BIOLOGY

Promoting Tumorigenesis by Suppressing Autophagy

Itay Koren and Adi Kimchi

Autophagy controls cellular homeostasis by degrading long-lived proteins, protein aggregates, and defective organelles. It also suppresses tumorigenesis by limiting inflammation, eliminating toxic unfolded proteins, and removing damaged mitochondria that produce reactive oxygen species (which damage DNA). Loss of these protective events could promote cancer initiation (1, 2). Support for the tumor suppressive function of autophagy emerged from the findings that the gene encoding the essential autophagic protein Beclin 1 functions as a haplo-insufficient tumor suppressor in mice and humans (3–6). However, a comprehensive mechanistic view of how autophagy is turned off during tumor development and, more specifically, whether the tumor-suppressive activity of Beclin 1 results from its canonical autophagic function, was still missing. On page 956 in this issue, Wang *et al.* (7) establish a connection between Beclin 1 and the Akt signaling pathway, which controls a large spectrum of cellular functions associated with cancer ranging from cell proliferation and survival to angiogenesis and metabolism. The finding underscores the importance of autophagy in tumor suppression (8).

Akt can block autophagy by phosphorylating, and thereby inhibiting, two targets: the tuberous sclerosis complex 2 (TSC2) and the proline-rich Akt substrate of 40 kD (PRAS40) (7). Both targets block the protein mTOR; when active, mTOR inhibits autophagy. In a search for additional direct substrates of Akt that are part of the core autophagic machinery, Wang *et al.* identified Beclin 1 as a target of Akt. The authors show that endogenous Akt associates with Beclin 1 in cultured human epithelial cells and phosphorylates it on specific sites (Ser²⁹⁵ and Ser²³⁴). Phosphorylation by Akt generated binding sites for the

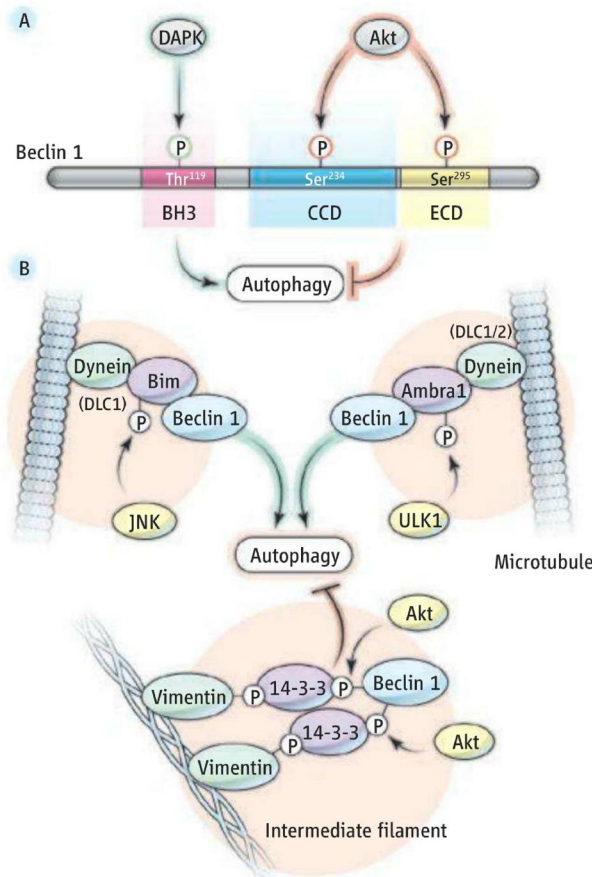
protein 14-3-3, which enhanced the binding of Beclin 1 to 14-3-3 and to the cytoskeletal protein vimentin. Sequestration of Beclin 1 to the cytoskeleton reduced its interaction with Vps34, a lipid kinase involved in autophagy nucleation. Consequently, Vps34 activity decreased, resulting in reduced autophagy. Notably, mutation of the Akt-specific phosphorylation sites in Beclin 1 that increased autophagy also suppressed different features of Akt-induced tumorigenesis such as anchorage-independent cell growth in culture

Tumorigenesis is controlled by the sequestration of an autophagy machinery component to the cytoskeleton.

and tumor growth in immunodeficient mice. These observations establish a link between the autophagic and the tumor-suppressive functions of Beclin 1, both of which are regulated through specific phosphorylation by Akt. To further strengthen these causative relationships, it will be necessary to examine whether the tumor-suppressive activity of the nonphosphorylated Beclin 1 mutant can be reversed by disrupting the autophagy pathway, for example, by simultaneously reducing the expression of downstream autophagic genes in this Akt-driven tumorigenesis system.

Two interesting paradigms on the function and mode of regulation of Beclin 1 are currently emerging. Beclin 1 activity appears to be tightly controlled, either negatively or positively, by signaling kinases with opposing functions in cells. It has been previously reported that DAP kinase (DAPK), a stress-induced kinase, phosphorylates Beclin 1 within its BH3 domain (position Thr¹¹⁹) and that this modification prevents binding of Beclin 1 to its inhibitor, Bcl-2/Bcl-x_L, thus promoting its autophagic activity (9). By contrast, the phosphorylation of Beclin 1 at distal sites by the cell growth-promoting kinase Akt suppresses the autophagic activity of Beclin 1 by controlling its intracellular localization (see the figure). Whereas Akt is a potent oncogene that is activated in tumors, the opposing kinase, DAPK, is a tumor suppressor subjected to loss or inactivation in many tumors (10), thus providing an additional correlative link between Beclin 1 activity and tumorigenesis. It would be interesting to screen human tumors that did not lose Beclin 1 or DAPK expression for mutations in these phosphorylation sites—either those that mimic Beclin 1 phosphorylation by Akt, or those that eliminate phosphorylation by DAPK.

Another emerging paradigm relates to sequestration of Beclin 1 to different cytoskeletal elements as a mechanism to control its func-



Phosphorylation and sequestration. (A) Phosphorylation of Beclin 1 with the indicated kinases either promotes or inhibits autophagy. (B) Beclin 1 is inactive when it associates with the dynein motor [dynein light chain 1/2 (DLC1/2)] and the microtubule cytoskeleton. Phosphorylation of Bim by JNK or of Ambra1 by ULK1, releases Beclin 1 and activates autophagy. Akt phosphorylates Beclin 1, allowing the adaptor protein 14-3-3 to link it to the cytoskeleton (vimentin and intermediate filaments). This blocks autophagy. P, phosphorylation; BH3, Bcl-2 homology 3 domain; CCD, coiled coil domain; ECD, evolutionarily conserved domain; JNK, c-Jun N-terminal kinase.

Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel. E-mail: itay.koren@weizmann.ac.il; adi.kimchi@weizmann.ac.il

tion. Sequestration of Beclin 1 to microtubule elements was known to block autophagy (11, 12). In one case, Bim, a BH3-only member of the Bcl-2 family, interacts with Beclin 1 and links Beclin 1 to the microtubule element, dynein light chain 1 (DLC1, also called LC8). The mislocalization of Beclin 1 to the dynein motor complex inhibits autophagy. A stimulus for autophagy was suggested to induce Bim phosphorylation, leading to the dissociation of Bim and Beclin 1 from DLC1 and autophagy induction (11). In another scenario, Beclin 1 is tethered to the microtubule cytoskeleton through an interaction with Ambra1, a protein that associates with DLC1/2. When the protein kinase

ULK1 is activated at the initiation stage of autophagy, it phosphorylates Ambra1; Beclin 1 is subsequently released from the dynein motor and becomes available to promote autophagy (12). In these two examples, regulation takes place through phosphorylation of Beclin 1–interacting proteins, which leads to activation of Beclin 1 and autophagy. Wang *et al.* document a quite different mechanism in which Akt phosphorylates Beclin 1 itself, promoting Beclin 1 sequestration to the cytoskeleton and suppressing its function in autophagy.

Several modes of Beclin 1 regulation appear to operate through its binding to different cytoskeletal elements. The mechanism

elucidated by Wang *et al.* may have important implications in tumor development.

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CHEMISTRY

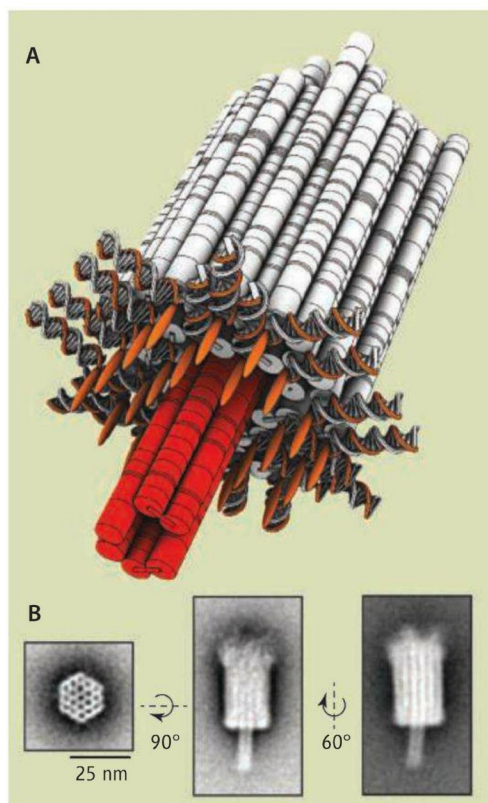
Functional DNA Origami Devices

Michael S. Strano

DNA is central to the existence of all life on Earth and uniquely encodes the instructions for producing such life. But it has another set of remarkable properties shared by a much larger class of molecules, most of which are yet to be invented. These “chemically sequenced polymers” (CSPs) are built by adding distinct monomers one at a time and can be programmed in such a way as to assemble into arbitrarily complex and three-dimensional shapes. Nature is far ahead of us in the creation and utility of such molecules, but on page 932 of this issue, Langecker *et al.* (1) provide a glimpse of the structural precision and functionality they offer for programmed assembly.

When the monomer units of a CSP are nucleic acids, the engineering of such structures is called DNA origami (2, 3) after the Japanese art of paper folding. Here, different parts of a long single-stranded DNA segment are brought and “stapled” together using smaller oligonucleotides that are programmed to hybridize at key locations in the structure. In this way, one can staple and fold the system into any three-dimensional shape.

CSPs are also used in protein folding and engineering, where amino acids are the sequencing elements; however, creation of programmable, functional devices with amino acids would require the ability to pre-



Advances in programmed DNA assembly are beginning to yield highly precise functional devices.

Membrane docking channel from DNA origami. (A) Cartoon of the DNA origami channel reported by Langecker *et al.* The channel has two parts, an extramembrane mouth (gray/orange) and a hydrophobic stem (red) extending into the membrane and can control the transport of a range of molecules. (B) Channel structure in averaged negative stain TEM images (1).

To underscore the point, Langecker *et al.* report a stunning advance toward programmable structure and function. Using DNA origami, they have designed and synthesized a working ion channel that can spontaneously assemble into a lipid bilayer. The design is derived from α -hemolysin (a protein that is excreted by *Streptococcus* bacteria to perforate a target cell, causing it to leach iron through the resulting channel for the bacterium to consume) (6). In the resulting device (see the figure), one part of the DNA is folded into a hydrophobic barrel that inserts itself into a nearby lipid bilayer membrane. An extramembrane portion on top of the barrel forms the mouth of the channel.

The level of complexity and function of this device is remarkable, as the TEM images and electrochemical measurements confirm. Once immobilized in a target membrane, the ion channel demonstrates gating behavior when an electrical potential is placed across it. The observed stochastic fluctuations in ion current resemble those seen in many biological (6) and synthetic (7) ion channels. The channel can also transport and recognize

dict protein folding under different condition. The original attempts to create synthetic CSPs are simplistic by comparison, but the field of multiblock polymers (4) highlights how elegant order can be generated from just a handful of sequenced “bases” represented by the distinct polymer blocks. Scientists are only just beginning to understand self-assembly and the connections between these various materials (5).

Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. E-mail: strano@mit.edu

DNA hairpins passing through the pore and distinguish differing DNA lengths; furthermore, it has impressive stability commensurate with that of biological ion channels. The possibilities with this system are endless, because one can vary the sequence and corresponding structure quite easily, analogous to site-directed mutagenesis in biology.

A CSP such as DNA also allows one to create nanoparticles and nanowires with broken symmetry—placing chemical groups at specific locations, as opposed to uniformly about an axis of symmetry—because each site of the sequence is addressable. This is no small task. An organic chemist knows how to brominate, say, a specific position on a benzene ring. But symmetry breaking of nanostructures is nearly impossible using existing methods. Yet it is the key to introducing the complexity necessary to create nanomachines with any sophistication.

Acuna *et al.* recently achieved such symmetry breaking (8). They constructed a DNA

origami scaffold that allows the precise placement of two equally sized plasmonic Au nanoparticles on either side of a DNA nanopillar. Optical excitation of the particle pair amplifies the local electromagnetic field hundreds of times. The resulting “nanoantenna” can record the fluctuations of single dye molecules, as well as the binding and unbinding of probe DNA segments. In this example, the broken symmetry of the scaffold, made possible by the sequence-addressable boundaries in DNA origami, enables the colocalization of several components in a way that offers precision, function, and utility.

It is not surprising that DNA is the first system for which humans have realized the potential of CSPs. Powerful tools such as the polymerase chain reaction and DNA sequencing provide unprecedented ability to program, synthesize, and error-check DNA sequences. Biotechnology has increased the production scale and lowered the cost of all forms of DNA and RNA. These advances

were driven, in large part, by the special function of DNA as a biological information carrier. A spoil of that enormous effort is the ability to create nanomachines of exceeding complexity. DNA origami and the larger yet unrealized field of chemically sequenced polymers promise a vast array of amazingly programmed structures, of which Langecker *et al.* and Acuna *et al.* have given us the first of what will be many examples.

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IMMUNOLOGY

Cooperative Transcription Factor Complexes in Control

Gustavo J. Martinez and Anjana Rao

The cornerstone of transcriptional regulation is the binding of transcription factors to regulatory elements in DNA. The fine-tuning of this process is almost universally achieved through the formation of “enhanceosomes,” assemblies of DNA-binding proteins that are stabilized by an intricate network of protein-protein and DNA-protein contacts. The power of such cooperative interactions is now highlighted by four recent studies (1–4), including the report by Glasmacher *et al.* on page 975 in this issue. Together, these studies demonstrate that a cooperative complex formed by a monomer of the interferon regulatory factor (IRF) family of transcription factors and a heterodimer of the activator protein–1 (AP-1) Jun and B cell activating transcription factor (BATF) has a major role in lineage specification and function in cells of the immune system.

The elegance of the enhanceosome strategy lies in its flexibility. Because the affinities of individual protein-protein and protein-DNA interactions tend to be rather low,

any given regulatory region in DNA can potentially assemble multiple factors whose recruitment is influenced by whether they can bind to nucleosomal DNA and recruit chromatin-remodeling complexes (“pioneering” transcription factors) or whether they prefer nucleosome-depleted DNA. The formation and stability of such a multiprotein-DNA complex depends on the concentrations of the DNA-binding proteins in the nucleus, their affinities for DNA sequences located within the regulatory region, and their ability to make cooperative interactions with other transcriptional regulators bound to adjacent DNA sequences (5).

In cells of the immune system—B cells, dendritic cells, and macrophages—IRF4 and IRF8 drive lineage-specific gene expression through cooperative interactions with the Ets transcription factor family on Ets-IRF composite elements (EICEs) (1, 6). These complexes are less likely to form in T cells, which express low amounts of Ets factors. IRF4 facilitates the differentiation of CD4⁺ T helper (T_H) cells into distinct functional subsets: T_H17 cells that produce the proinflammatory cytokine interleukin-17

The cooperative binding of transcription factors regulates the differentiation of T cells as well as the generation and function of B cells and dendritic cells.

(IL-17); follicular helper T cells required for antibody production by B cells; and T_H2 cells that mediate immune responses to parasites (7–9). Because IRF4 binds to DNA with relatively weak affinity (6), its activity in CD4⁺ T cells suggests a requirement for cooperative interactions with other transcription factors. The identification of the BATF-Jun heterodimer as a cooperative factor has now been established through chromatin immunoprecipitation followed by next-generation sequencing (ChIP-Seq) (1, 2, 4). Bioinformatic analyses of the ChIP-Seq data identified two DNA motifs, referred to as AP-1–IRF composite elements (AICEs) (see the figure).

To experimentally confirm the bioinformatic analyses, the studies show that binding of BATF to composite sites decreased in T cells lacking IRF4, and vice versa (1, 2, 4). Similarly, electrophoretic mobility shift assays (EMSAs) demonstrated the cooperative binding of BATF-Jun heterodimers with IRF4 (or IRF8) to composite elements. Thus, IRF4 and IRF8 are distinct from the other more widely expressed IRF family members, not only in their expression pattern in

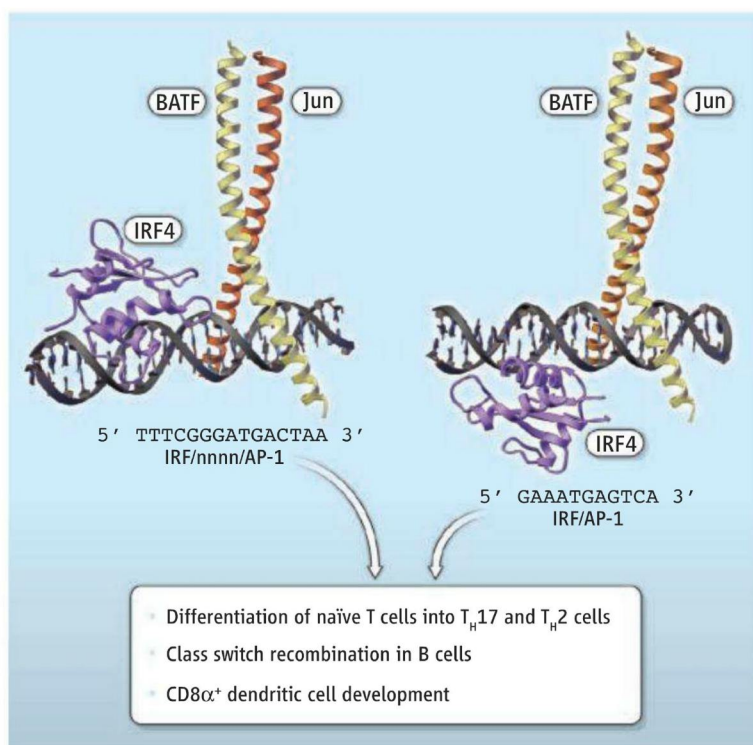
La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037, USA. E-mail: arao@liai.org

The structure of cooperation. A model of the BATF-Jun-IRF4 complex (2) is shown, based on the known structures of the Jun-ATF2-IRF3B complex in the interferon- β enhancosome (14) and the PU.1-IRF4 complex on the *Ig* λ gene enhancer (15). The model was built using AICE motifs from *Ctla4* (4 bp spacing) and *Bcl11b* (0 bp spacing) loci. The sequences of the *Ctla4* (left), and *Bcl11b* (right) AICE elements are shown. The different outcomes of cooperation between IRF4 and BATF-Jun in the immune system are listed.

immune cells but also in their cooperative binding to AP-1 and Ets family members on AICE and EICE motifs, respectively (1, 3).

The transcription factor signal transducer and activator of transcription 3 (STAT3) may also be part of an extended IRF-BATF-Jun complex. STAT3 binds to Jun (10) and the BATF-Jun-IRF heterotrimer could potentially recruit activated STAT3 to relevant target genes. There was no enrichment for STAT3 binding sites in the immediate vicinity of AICE composite elements, suggesting that STAT3-Jun association could occur between proteins bound to spatially separated sites. Indeed, STAT3 and IRF4 both bind to the *Prdm1* promoter element (11), but at nonadjacent sites.

To understand the regulatory network driving T_H17 cell differentiation, Ciofani *et al.* (4) used a comprehensive systems biology approach in which they combined ChIP-Seq for several transcription factors implicated in T_H17 differentiation with transcriptional profiling of cells expressing or lacking these factors. Among the factors tested, expression of BATF and IRF4 was induced by T cell receptor stimulation alone, whereas the expression/activation of transcription factors Maf, STAT3, and retinoic acid receptor-related orphan receptor gamma t (ROR γ t) was induced only if the T cell receptor was stimulated in the presence of the T_H17 -polarizing cytokines transforming growth factor β (TGF β) and IL-6. In nonpolarizing conditions, BATF and IRF4 bound to DNA cooperatively even in the absence of the other three T_H17 transcription factors. The authors propose that BATF and IRF4 function as pioneer transcription factors that nucleate the binding of lineage-specific transcription factors to DNA by promoting



chromatin remodeling and nucleosome repositioning (4). In addition, expression of the AP-1 family member Fos12 was induced in T_H17 cells and repressed IL-17 production (1, 4). T cells lacking Fos12 showed increased IL-17 production and expression of Foxp3, the transcription factor essential for regulatory T cell function. Ciofani *et al.* demonstrate substantial overlap between BATF and Fos12 binding sites in DNA; Glasmacher *et al.* found that the Fos12-JunB heterodimer is unable to form a cooperative complex with IRF4 on AICE motifs. Together, these data suggest that BATF and Fos12 might compete for DNA binding to AICE motifs, and that Fos12 may antagonize BATF and repress IL-17 production by binding to the motifs but preventing IRF4 recruitment. Thus, the balance of BATF versus Fos12 occupancy at AICE sequences would dictate the amount of IL-17 produced.

The *in vivo* importance of the BATF-IRF4 interaction was addressed in depth by Tussiwand *et al.* (3). The authors show in mice that BATF1, BATF2, and BATF3 display redundant functions, as might have been expected based on similar DNA target sequences. BATF-IRF4 complexes regulate dendritic cell development, B cell antibody class switching, and T_H17 cell differentiation *in vitro* and *in vivo*. In EMSA assays and reconstitution experiments *in vitro*, four residues in BATF were identified as crucial for BATF-Jun-IRF4 complex formation (1, 3).

The four independent studies reemphasize the physiological importance of cooperative interactions between unrelated transcription factors on DNA. The pairing of strong (or moderately strong) and weak binding sites in DNA is typical of composite elements. For example, the transcription factor nuclear factor of activated T cells (NFAT) binds to a relatively strong consensus site whereas the AP-1 site is typically nonconsensus. Nevertheless, the cooperative NFAT-AP-1 complex binds with high affinity to DNA (12, 13). The advantages for gene regulation and signal integration are obvious: The activity of cooperating transcription factors can be regulated at both transcriptional and posttranscriptional levels in response

to signaling inputs, allowing enormous flexibility and control over gene expression. Future research will illuminate the structural basis for cooperative BATF-Jun-IRF binding to the two classes of AICE composite sites, and define by affinity and kinetic measurements the precise increase in the stability of the cooperative complex over that of the individual AP-1 and IRF components alone.

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MATERIALS SCIENCE

Speeding Up Artificial Muscles

Mark Schulz

Artificial muscles—materials that change size or shape when activated by some stimulus—are attractive for applications in robotics and in sensing and control technologies because they provide large force and motion while being compact and lightweight. Currently, artificial muscles (and smart materials in general) are limited in the combinations of force, motion, and speed that they can generate. Also, their efficiency may be low, or they may need to operate in a certain environment, such as within an electrolyte. On page 928 of this issue, Lima *et al.*

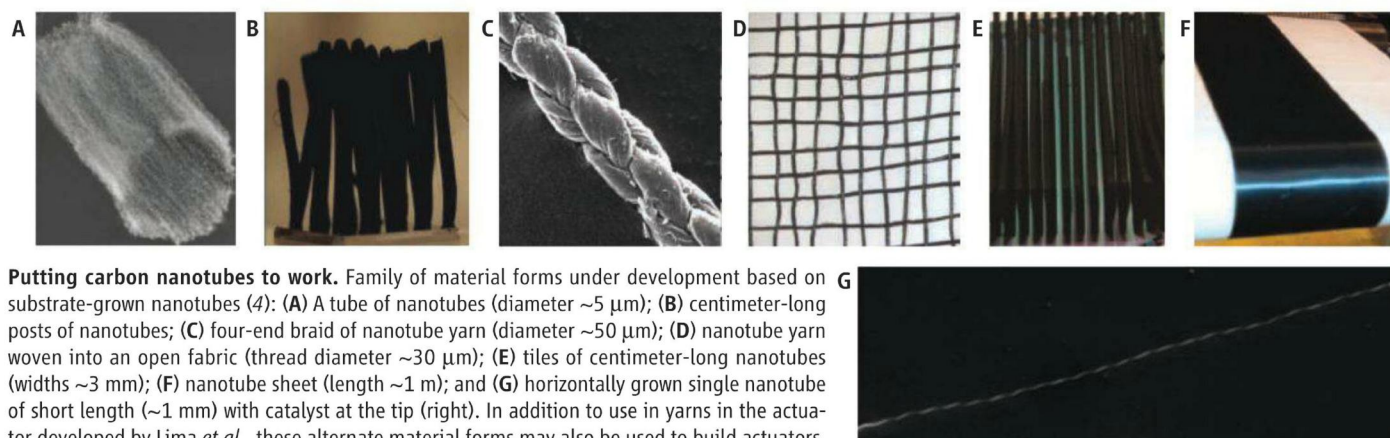
is similar to cotton yarn but much smaller in diameter and much stronger and can be over-twisted and coiled to store energy for reversible actuation. The muscle is actuated by heating, which causes a phase transition and volume expansion of the guest material. Actuation is likewise possible with guest materials that absorb chemicals and change volume. Thermal contraction of the coiled nanotube material also produced actuation.

Good electrical conductivity of the nanotube yarn makes heating and actuation easy to control. The high surface area and high

The twisting motion of carbon nanotube yarns is driven rapidly by the melting and expansion of a surrounding solid wax.

such as rotation and contraction speed and forces generated by the muscle. The muscles actuate with larger motion in torsion than in contraction, and operate in air, but can also operate in vacuum at high temperature for higher performance. Artificial muscle can also act as an intelligent sensor that detects environmental conditions such as the presence of a chemical or a change in temperature. The sensors provide either a reversible or a nonreversible linear or rotary response.

Despite the advances in performance reported by Lima *et al.*, artificial muscle



Putting carbon nanotubes to work. Family of material forms under development based on substrate-grown nanotubes (4): (A) A tube of nanotubes (diameter $\sim 5\ \mu\text{m}$); (B) centimeter-long posts of nanotubes; (C) four-end braid of nanotube yarn (diameter $\sim 50\ \mu\text{m}$); (D) nanotube yarn woven into an open fabric (thread diameter $\sim 30\ \mu\text{m}$); (E) tiles of centimeter-long nanotubes (widths $\sim 3\ \text{mm}$); (F) nanotube sheet (length $\sim 1\ \text{m}$); and (G) horizontally grown single nanotube of short length ($\sim 1\ \text{mm}$) with catalyst at the tip (right). In addition to use in yarns in the actuator developed by Lima *et al.*, these alternate material forms may also be used to build actuators.

(1) describe a faster and longer-stroke artificial muscle based on yarns made from sheets of carbon nanotubes in which liquid electrolytes are replaced with a solid guest or filler material such as wax; melting and solidifying the wax twists or untwists the yarn and generates motion. Other guest materials are activated by chemical absorption or illumination by light. The new artificial muscle outperforms existing artificial muscles (2), allowing possible applications such as linear and rotary motors, and might replace biological muscle tissue if biocompatibility can be established.

Lima *et al.* built their artificial muscles by first growing a vertically aligned forest of carbon nanotubes, from which they drew a thin sheet of nanotube bundles. They deposited wax filler on the sheet and then twisted it to form a yarn (the wax could also be melted and integrated into prepun yarn). The yarn

interfacial energies of the nanotubes and the pores in the yarn contain the guest, which is an aspect peculiar to the nanoscale. In a macroscale yarn (a rope), wax filler would extrude under loading, and the actuation effect would be small.

Melting expands the wax, and the helical architecture of the yarn causes the volume change (swelling) of the wax to apply radial and axial forces that can twist or untwist it and change its length. The yarn is pliable and supports only tension loading (it can pull, but not push). The forces generated are limited by the need to keep the guest within the yarn and by the tensile strength of the yarn material. The overall interaction between the guest expansion and yarn is quite complex; a supplementary figure in Lima *et al.* (figure S11) shows how volume increase or decrease can cause tensile contraction or extension depending on the initial twist angle for an individual ideal single helix.

Several designs of yarn pretwisting and constraint define the actuation properties

design can still be improved. One tactic is better constraint of the volume expansion of the guest material; it may be possible to open the ends of nanotubes and fill them with guest material. The average tensile stresses are still low and comparable to those of macroscopic carbon fibers. Ideally, artificial muscle should approach the strength of individual perfect nanotubes, and some researchers (3, 4) maintain that the extraordinary properties of nanotubes will eventually be scaled up and provide a new ultimate material useful for reinforcement, actuation, and sensing.

Carbon nanotubes usually grow normal to their substrate, which often limits their length, so like macroscopic yarns, long strands are woven together from many shorter fibers held together by twisting. Efforts are being made to scale up horizontal synthesis of carbon nanotubes. Optimizing synthesis conditions (which largely is a process engineering problem) could eliminate junctions between nanotubes in the yarn, which would greatly increase strength and toughness. Horizontal

Mechanical Engineering, University of Cincinnati, Cincinnati, OH 45221-0072, USA. E-mail: mark.schulz@uc.edu

growth of nanotubes as long as 10 cm has recently been reported (3). Forests of 2-cm-long nanotubes have also been produced, and the quality is being improved by annealing (4). These recent results point to there being no physical barrier to growing long nanotubes, as was anticipated by Smalley (5).

Another issue is the role of defects in nanotubes; even a few defects can greatly reduce nanotube strength (i.e., weak links in a chain). Thermal annealing can substantially heal the defects to bring the nanotubes to near pristine condition (6). Finally, commercial applications of actuators might require use of different architectures. Thus, a practical area of improvement would be to expand the number of forms of nanotube materials that can meet this need. Fortunately, nanotube tubes, posts, braids, fab-

rics, tiles, and sheets (see the figure) are already under development (4). For example, braiding yarn increases energy storage (7), and fabrics and sheets allow two-dimensional actuation. Further work could study the mechanics and failure mechanisms of the muscle, including how elastic modulus changes with actuation and characterizing extrusion of the guest, and how, at higher stress, nanotubes slide in the yarn.

The artificial muscle developed by Lima *et al.* is also a step toward commercialized devices. Applications of nanotube-based actuators include sensors for the environment, aerospace materials, carbon machines (1–4), nanotextiles (7), and nanopropulsion (8). Artificial muscle also serves as a prime example of how changes in mechanics at the nanoscale can enable invention.

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EVOLUTION

Convergent Evolution of Hearing

Ronald R. Hoy

How do human ears work? The textbook explanation starts by dividing the ear into three separate anatomical entities that have equally separate functions in converting airborne sound (pressure waves outside the ear) into fluid-borne traveling waves inside the ear, a conversion that makes long-distance hearing on dry land possible (1, 2). On page 968 of this

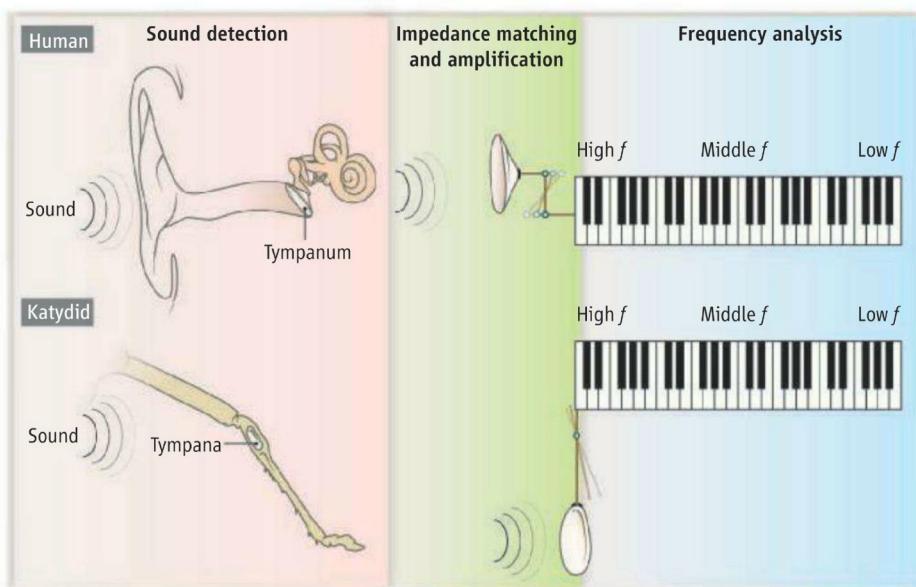
issue, Montealegre-Z. *et al.* (3) show that although the hearing organ of a rainforest insect looks very different from a human ear, it can be divided into the same three functional entities, providing evidence for convergent evolution.

In the first step of human hearing, airborne sound waves arriving at the outer ear cause the tympanic membranes/eardrums to vibrate. The eardrum is anatomically coupled to a trio of interlocked, delicate ear bones (hammer, anvil, and stirrup) that

The functional and anatomical aspects of hearing in a rainforest insect are remarkably similar to those in humans.

comprise the middle ear. These bones convert the airborne vibrations of the eardrum into fluid-borne vibrations in the cochlea, wherein our acoustic detector hair cells are bathed and reside. It is through these tiny ear bones that the extreme inefficiency of transferring the intensity of airborne waves through fluid is overcome through a process called impedance matching. In effect, the middle ear bones act as a lever that mechanically couples the eardrum to the cochlea (see the figure). This lever action converts

Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853, USA. E-mail: rrh3@cornell.edu



Convergent evolution of the hearing mechanism in humans and katydids. In the detection stage (pink panel), airborne sound stimulates the tympana, which drive an energy transfer mechanism (green panel). In both humans and katydids, this coupling mechanism efficiently transfers and amplifies vibrational energy from air to fluid, solving the problem of impedance mismatch. Montealegre-Z. *et al.* now show that an equivalent mechanism operates in katydids. In both cases, it involves a system of mechanical levers (brown bars/blue circles = fulcrums/pivot points). The fluid-filled inner ear contains a linear array of auditory receptor cells, represented here by the keys of a keyboard; this is where frequency analysis is performed (blue panel). In humans, this auditory mechanism has been known for nearly a century and thought to be special to humans. Montealegre-Z. *et al.*'s discovery of a closely analogous mechanism in katydid ears is a triumph of the power of micromechanics and nano-optics technology applied to minute biological specimens.

and amplifies large-amplitude vibrations of the eardrum into smaller but more forceful vibrations that are conducted through the fluid of the cochlea as a traveling wave. Finally, the traveling waves are converted into bioelectric signals in auditory sense cells in the inner ear, leading to excitation of the auditory nerve itself. In the cochlea, the sensory hair cells are distributed as an orderly linear array along the length of the cochlear membranes. Each cell in the linear array of receptors responds to a specific pitch/frequency of a pure tone according to its location along the array, with low frequency cells at one end, high frequency cells at the other, and intermediate frequency cells between them (see the figure) (4); this is the principle of tonotopic organization (1, 2).

Montealegre-Z. *et al.* now report that the functional and anatomical aspects of hearing in humans find extraordinary similarity in the ear of a rainforest katydid. They show that in the latter, outer-ear tympanal membranes are coupled to a stiff, leverlike middle ear-like structure, which is in turn coupled to an elongated, fluid-filled chamber of the inner ear that contains a linear array of sensory receptors (see the figure). The key finding is the impedance matching and amplification step by a leverlike, middle ear-like component, the tympanal plate (TP), which acts in concert with the tympanal membranes (TM) that heretofore had not been known to exist. This TM-TP “middle ear” efficiently transfers airborne vibrations into vibrations of the fluid-filled acoustic vesicle.

These findings were made possible by the use of an array of state-of-the-art technologies: x-ray microtomography, which allows high-resolution imaging of fresh animals or specimens, microscanning laser-Doppler vibrometry (DLV), which allows measurement of sound-induced tympanal and cuticular vibrations at high spatial resolution, and 3D reconstruction imaging software applied to the microtomography (micro-CT) data. The micro-CT permits imaging of a stationary live insect (or appendage) while the x-ray tube and detector rotate around it. It would have been extremely difficult to reveal the microscale anatomical features and measurement of their mechanical responses to sound using conventional microscopy.

The parallelism in anatomy and function is the result of convergent evolution between the ears of humans and katydids. It is as surprising as it is remarkable and has important implications for compara-

tive auditory research. Katydids belong to a large suborder of acoustically active insects, the Ensifera, which includes the crickets (5). The tympanal mechanics (6) and even the linear tonotopic distribution of sensory cells (7) in these insects have been known for decades, but the manner in which tympanal mechanics generate linear tonotopy has been debated. The work of Montealegre-Z. *et al.* opens the way for re-examining previous results to see if a similar TM-TP mechanism occurs widely within the Ensifera.

Given the discovery of such an unexpected hearing anatomy in an insect, it may be valuable to revisit the phylogenetic spread of sensitive hearing and frequency tonotopy not only in insects but across all invertebrates. Insect ears are much more diverse than those of vertebrates, in location on the body as well as in form and function. The three compartment hearing organ in terrestrial vertebrates, including mammals, evolved out of the developmental imperatives surrounding the evolution of the vertebrate ear on the head (8). How-

ever, no such constraints apply to the hearing organs of insects (9). In fact, three-compartment hearing organs are likely to have evolved first in insects, and only much later in mammals. Moreover, all insect ears are miniscule organs, compared to those of vertebrates. The miniature ears of insects may provide valuable insights for developing the next-generation of auditory biosensors.

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ARCHAEOLOGY

Mediterranean Island Voyages

Alan Simmons

Archaeological studies show that humans reached Mediterranean islands much earlier than previously thought.

Some of the classical world's most innovative cultures developed on Mediterranean islands, but their earlier human use is poorly known. The islands, particularly those further from the mainland such as Crete and Cyprus, were thought to have been first colonized about 9000 years ago by late Neolithic agriculturalists with domesticated resources. Until about 20 years ago, claims of earlier, pre-Neolithic occupations on any of the islands did not stand up to critical scrutiny (1), but current investigations are challenging these perceptions. Discoveries on Cyprus, Crete, and some Ionian islands suggest seafaring abilities by pre-Neolithic peoples, perhaps extending back to Neanderthals or even earlier hominins. In Cyprus, Neolithic sites have been documented that are nearly as early as those on the mainland.

Evidence for early seafaring by pre-

Neolithic humans comes from several parts of the world. For example, pre-Neolithic people must have been able to cross substantial expanses of sea to reach Australia by at least 50,000 years ago (2). Additionally, findings from the Indonesian Wallacea islands suggest the presence of hominins as early as 1.1 million years ago on Flores Island (3). If correct, the latter suggests that *Homo erectus* had considerable seafaring and cognitive skills, because even at times of lower sea levels, these areas were separated from the mainland by substantial amounts of open sea.

In the Mediterranean, the early-seafaring debate has focused on three time periods (4). “Deep time” claims for Paleolithic occupations have been particularly controversial and not well documented because they involve hominins that potentially predate *Homo sapiens*. Evidence for occupation around 12,000 years ago, immediately before the Neolithic, was somewhat more convincing but was restricted to islands that were either close to the mainland or may have been con-

Department of Anthropology, University of Nevada, Las Vegas, Las Vegas, NV 89154, USA. E-mail: simmons@unlv.nevada.edu

nected to the mainland at the time. Some of the strongest, if indirect, evidence for seafaring was the occurrence of obsidian from the Aegean island of Melos at the mainland Greek coastal site of Franchthi cave, beginning from the 11th millennium before the present (B.P.) (5, 6). The third period relates to early Neolithic settlement. Overall, claims of occupation for all three time periods were poorly supported by empirical data.

Recent studies are beginning to provide better evidence for exceedingly ancient human occupations. On Crete, for example, artifacts that include quartz hand axes, trihedral picks, and cleavers have been interpreted as Lower Paleolithic, based on their typology and association with geological deposits that may date to ~170,000 years ago (7). Research on the southern Ionian islands suggests a human presence as early as ~110,000 years ago (8); these islands are closer to the mainland than Crete is, but geological evidence indicates that they were not connected to the mainland. The implications of these findings are substantial, since the hominins responsible would be either Neanderthals or perhaps even *Homo erectus*. Given the distance of Crete from the mainland and no evidence for a land bridge, this voyage was no small feat.

Excavations at Akrotiri *Aetokremnos* on the southern coast of Cyprus (see the figure, panel A) (9, 10), have also provided firm evidence for occupation during the period immediately before the Neolithic (the Akrotiri Phase). *Aetokremnos* has stratigraphic context that facilitates dating, distinctive artifacts including “thumb-nail” scrapers and other tools, a huge faunal assemblage consisting mainly of the endemic dwarf pygmy hippopotamus (*Phanourios minutus*), and an absolute chronology supported by more than 30 radiocarbon dates indicating an occupation around 12,000 calibrated years B.P. This is about 3000 years earlier than previous evidence for the earliest occupation of Cyprus (the Neolithic Khirokitia culture).

Evidence is also accumulating for other Mediterranean island sites roughly contemporary with the Akrotiri Phase. In Cyprus, for example, these include coastal sites (Nissi Beach, Aspros) and an interior site (Roodias); elsewhere in the Mediterranean, there are sites of a similar age on Lemnos (Ouriakos) and Crete (Damnoni) (11–13). Preliminary results show that some of the artifacts found resemble those at *Aetokremnos*. All of the sites mentioned above, however, have only recently been investigated, and dating and other information is still limited.

New data are also changing the way the



Early occupation. Sites such as Akrotiri *Aetokremnos* on the southern coast of Cyprus [(A), overview and hearth feature] are providing evidence for pre-Neolithic occupation of Mediterranean islands. Other sites, including PPNB Ais Giorkis [(B), overview and female stone figurine] and PNA Klimonas [(C), communal building and projectile point], demonstrate their occupation during the early Neolithic. The scale bars correspond to 0.5 m for the hearth, 10 cm for the figurine, and 5 cm for the projectile point.

Neolithic, with its more substantial physical remains, is viewed, and Cyprus is again at the forefront (14). Prior to recent studies, the earliest evidence for Neolithic occupation on the island was the Khirokitia culture. It showed few parallels with the mainland, having only the basic economic suite of key domesticated plants and animals. Cattle, an important economic and ritual animal on the mainland, were absent. This picture changed dramatically with the discovery of several sites termed Cypro-PPNB (Pre-Pottery Neolithic B), the oldest dating to ~10,400 B.P.

The sites are distinct from one another but resemble mainland PPNB sites with regard to chipped stone technology and the presence of villages and domesticated plants and animals (15, 16). Most are coastal, but one is upland (Ais Giorkis, see the figure, panel B). Three sites contain cattle bones. The diversity of the Cypro-PPNB sites is in marked contrast to the relatively homogeneous Khirokitia culture. It is likely that full-scale colonization of Cyprus occurred during the Cypro-PPNB (17).

The most recent discoveries in Cypriot

CREDITS: (PANEL A) ALAN SIMMONS; (PANEL B) REPRINTED WITH PERMISSION FROM (17); (PANEL C, MAIN PHOTO) J.-D. VIGNE/CNRS-MNH; (PANEL C, INSET) REPRINTED WITH PERMISSION FROM (19)

Neolithic archaeology are two sites dating to the Pre-Pottery Neolithic A (PPNA, ~11,700 to 10,500 B.P.). As on the mainland, the PPNA in Cyprus includes villages but does not yet contain morphologically domesticated plants and animals. These sites essentially erase the chronological gap between the Akrotiri Phase and the Neolithic. One site, Ayia Varvara *Asprokremnos*, is a small interior locality (18), the other, *Klimonas* (see the figure, panel C), a more substantial coastal settlement where plants were apparently cultivated but not domesticated (19).

The Neolithic transformation initially occurred in the Near East, but then spread to adjacent areas. This transmission is often thought to have been through Anatolia, but the new research also suggests maritime routes, with the Cypriot evidence indicating a substantial level of mainland interaction.

Genetic data also point to linkages between northern European Neolithic populations and modern groups that include Cypriots (20).

The past 20 years have revolutionized our understanding of the early occupation of the Mediterranean islands. Future research should include developing better chronological controls, conducting rigorous surveys, excavations, and analyses, searching for sites within an intact stratigraphy, and asking questions related to why early humans set forth to these islands. Such studies will continue to change our conceptions of early seafaring and the reasons behind it and of the ever widening influence of the Neolithic Revolution.

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PLANETARY SCIENCE

A Vitrage of Asteroid Magnetism

Benjamin P. Weiss

In the early solar system, some protoplanets experienced large-scale melting, leading to the formation of a metallic core overlain by a rocky mantle. This differentiated structure has persisted to the present day in large bodies such as Earth. In Earth's core, vigorous churning of molten metallic liquid generates the geomagnetic field in a process known as the dynamo (1). Although we have no samples of Earth's core, many smaller protoplanets were catastrophically shattered over the intervening eons, producing the present-day asteroid belt and providing us with meteorite samples from their deep interiors. These samples provide a natural cross section of asteroid interiors, with stony meteorites thought to have formed at shallow depths and iron meteorites in the core. Intermediate in composition between these two types are the pallasites, spectacular mixtures of translucent, gem-quality olivine crystals and iron-nickel metal that, when backlit, take on the quality of a medieval stained-glass window (vitrage) (see the first figure). The origin of pallasites and the nature of their parent body (2) have perplexed meteoriticists since the first, eponymous meteor-

ite Pallas was described in 1794 (3). On page 939 of this issue, Tarduno *et al.* (4) suggest that the pallasite parent body was the product of a near-catastrophic impact of a molten body onto a differentiated protoplanet with an active core dynamo.

Starting with the earliest visions of meteorite parent body interiors (5), it has been widely assumed that pallasites originated from a gradational boundary layer between the metallic core and overlying rocky mantle (6) (see the second figure). Nevertheless, it

A group of stony-iron meteorites record an ancient magnetic field that may reveal an origin far from the asteroidal core.

was soon recognized that olivine's relatively low density makes such a mixture buoyantly unstable (7). Furthermore, the diverse rates at which various pallasites cooled may be inconsistent with formation deep below an insulating mantle and anchored to a thermally conducting metallic core (8). These findings motivated alternative proposals that pallasites formed as the result of an impact onto a differentiated planetesimal that structurally scrambled the body by mixing molten core and solid mantle materials (8).

Tarduno *et al.* add a fascinating new twist to this debate with their discovery of remanent magnetization in two pallasites. When a rock cools in the presence of a magnetic field, it can acquire remanent magnetization whose intensity is proportional to the field. This magnetization can then persist for billions of years, long after the paleofield has dissipated. In this way, rocks from planetary bodies record the presence of past dynamo activity.

Because of their small size, asteroidal cores have long since solidified and therefore cannot be generating mag-



Pallasite vitrage. Photograph of the pallasite Esquel showing translucent olivine crystals suspended in metal.

Department of Earth, Atmospheric, and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. E-mail: bpweiss@mit.edu

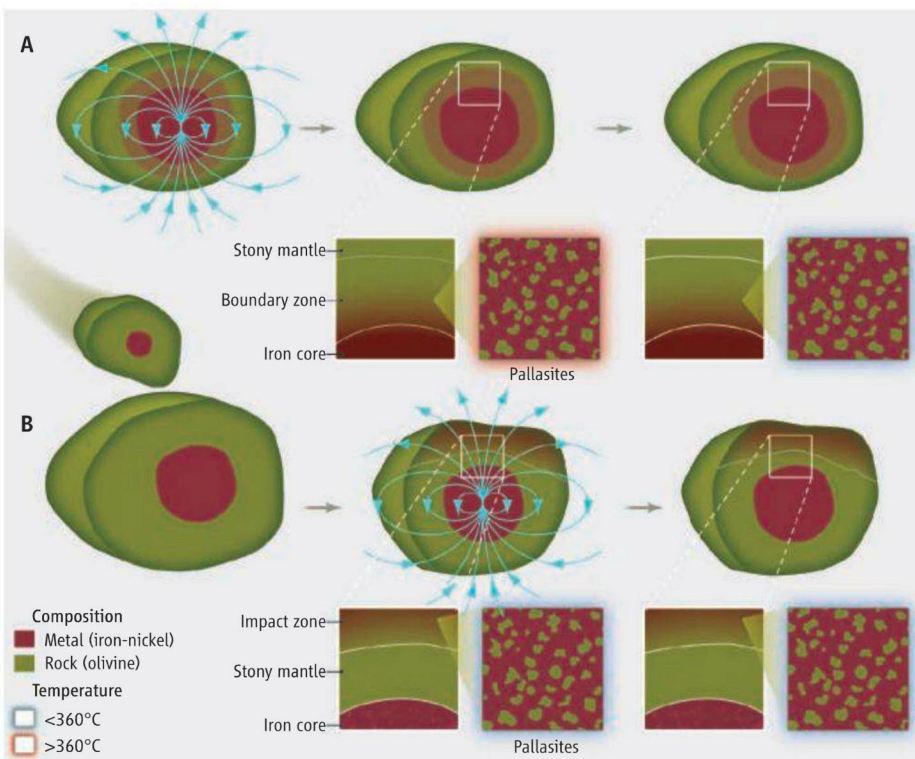
netic fields today. Until recently it had been unclear whether asteroids could ever have generated magnetic fields—even when their cores were molten—given that the feasibility of the dynamo process generally increases with core size. However, studies of several stony meteorite groups indicate that core dynamos probably operated in at least several early asteroids (9, 10), thus making asteroids the smallest objects known to have generated magnetic fields.

Meteoriticists have wondered whether iron or pallasite meteorites might record a core dynamo due to their assumed close proximity to the field-generating region of these bodies. However, these meteorites have stubbornly defied magnetic investigations since they were first attempted in 1959 (11). A chief difficulty has been that chunks of intergrown iron crystals typically have poor magnetic recording properties (12). Furthermore, because metallic cores should be mostly isothermal (because of their high thermal conductivity), by the time that core metal cools to temperatures sufficiently low that it could become permanently magnetized, any dynamo may have long since decayed away (13).

Tarduno *et al.* sidestep the first problem by analyzing extracted olivine crystals, which have far better magnetic recording properties than the surrounding metal. These pallasites record substantial magnetic fields, with intensities ranging up to nearly twice that of Earth today. Assuming that these fields were directly produced by an ancient dynamo in the pallasite parent body core, Tarduno *et al.* argue that the body could not have been too big (otherwise, the pallasites would have to come from near the surface, where they would be shattered or destroyed by meteoroid impacts) nor too small (or else they would have cooled faster than observed). They also argue that the pallasites could not have formed at the core-mantle boundary because of the aforementioned requirement that they must cool down before the cessation of dynamo activity. Rather, formation in the outer ~40 km of a protoplanet ~200 km in radius with a core 100 km in radius matches the combined constraints. As a result, they propose that pallasite metal did not come from the interior core of the protoplanet but rather from that of a differentiated foreign impacting body (see the second figure).

These paleomagnetic measurements are an important new data set with far-reaching implications for the origin of pallasites. A caveat is that it is unknown whether the field that magnetized these pallasites was that of an active dynamo or rather from remanent magnetization in surrounding rocks. For example, surface fields in some locations on Mars today are as strong as Earth's field even though Mars does not have an active dynamo (14). If this were true for the pallasite parent body, an earlier dynamo would still be indicated (because it would likely be required to have produced the magnetization in the surrounding rocks), but the pallasites' magnetism would not rule out an origin at the core-mantle boundary.

Among the terrestrial planets, only Earth and Mercury today have active dynamos. The pallasite parent protoplanet now becomes at least the fourth asteroid-sized body known to have generated a core dynamo, even though the magnetization of only a few meteorite groups has yet been analyzed in detail. This suggests that planetary dynamos may have at one time been common. One can imagine that the early solar system, which contained perhaps thousands of protoplanets larger than 100 km in diameter (15), once brimmed with swarms of little magnetospheres.



Pallasite production. Two models for the main-group pallasite parent body. (A) Pallasites formed in a gradual boundary layer between the metallic core and silicate mantle. The pallasites would not record an active dynamo field because they could not have cooled sufficiently to become permanently magnetized until after the dynamo had decayed. (B) Pallasites formed near the planetary surface as a result of metal injected into a protoplanet from the molten core of an impactor (4). They cooled while a dynamo was still active, thereby acquiring remanent magnetization.

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INTRODUCTION

Biomaterials

WHEN TRYING TO DEVELOP AN ARTIFICIAL SKIN, PROFESSOR KIM WOODHOUSE CAME up with several formulations based on an elastomeric polymer. One of them had the mechanical properties that most closely matched human skin, so she thought it would be the formulation the surgeon would want to test. The surgeon, however, came in, flopped each of the samples in his hands, and picked a different sample for surgical testing. That formulation became the standard for her lab.

Engineers think about formulas and numbers, surgeons work with their hands, physicists focus on explaining materials behavior, and biologists analyze complex cellular interactions. They all work in biomaterials, but they often speak different languages and have different priorities. For biomaterials to move from the lab to clinical use, these groups increasingly need to work together. With this in mind, *Science* and *Science Translational Medicine* are covering the basic, the applied, and everything in between—the so-called translational space.

Starting with the basics, Gonzalez-Rodriguez *et al.* (p. 910) review in *Science* how applied physics concepts learned from the study of the rheological properties of soft materials are being used to understand cell sorting and tissue mechanics. Such in vitro systems can help to answer basic biological questions, as explained in a *Science Translational Medicine* Perspective by Tibbitt and Anseth.

Biomaterials also drive forward technology development, as highlighted in two *Science* Reviews. Nanoparticles can enhance drug delivery and efficacy, yet a trade-off exists between functionality and complexity, which can dramatically affect the potential for regulatory approval. To this end, Cheng *et al.* (p. 903) explore finding the right balance in nanoparticle design. Printing and rapid prototyping can improve the potential clinical application of biomaterials. Derby (p. 921) contrasts these technologies, which are being used to fabricate tissues.

On the applied side of biomaterials, a major unmet clinical need is engineered whole organs. In *Science Translational Medicine*, Atala, Kasper, and Mikos review the latest advances in engineering complex tissues. It may be possible to combine cells and biomaterials into a structurally and functionally competent organ, but vascular networks are needed, as highlighted by Bae *et al.* in a related *Science Translational Medicine* Perspective. These issues are also raised in a *Science* Review by Huey *et al.* (p. 917), which discusses why more success has been realized in regenerating bone than cartilage. In a News story (p. 900), Hvistendahl spotlights China's push into the field of tissue engineering.

During all steps of translation, regulation and commercialization must be kept in mind. These aspects are central to the discussion in a *Science Translational Medicine* Review by Pashuck and Stevens on designing biomaterials for the clinic. In a *Science Translational Medicine* Commentary, the editors asked thought leaders from various backgrounds one question: What is the greatest regulatory challenge in the clinical translation of biomaterials? Several unifying themes emerged, including collaboration, cost, and the trade-off between innovation and time to approval.

We encourage readers to explore this diverse package of articles from *Science* and *Science Translational Medicine* and to broaden their view of the biomaterials world.

— MARC LAVINE, MEGAN FRISK, ELIZABETH PENNISI

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Science

NEWS

China's Push in Tissue Engineering

Valued for their innovation potential, biomaterials efforts in China prosper

NANTONG, CHINA—Mounted to the entrance of the Jiangsu Key Laboratory of Neuroregeneration is a plaque listing two dozen grants—a total of \$2.87 million over 18 years, impressive for a lab in the somewhat obscure Nantong University here, a few hours' drive from Shanghai. A sparkling 4000-square-meter complex outfitted with millions of dollars' worth of equipment, as well as a kitchen, recreation room, and locker and shower facilities, is an even greater testament to the success of the lab's director, Gu Xiaosong. An expert in peripheral nerve regeneration, he's pushed to make neuroregeneration “a distinguishing characteristic of our school.”

But in some regards, Gu's lab is not that unusual. It is one of dozens to capitalize on a recent Chinese government push in tissue engineering.

Chinese leaders see tissue engineering as a promising area for innovation, says Cui Fuzhai, a bone and brain tissue engineering specialist at Tsinghua University in Beijing: “They feel it is important to the economy—that it has clear applications.” In recent years, China's national grant programs have singled out this field, along with other aspects of biomaterials research,

for growth. From 1999 to 2009, according to a recent analysis by the Boston-based technology consulting firm Lux Research, more than \$80 million from various government channels went to tissue engineering and stem cell research in the country. While the government tends not to release funding totals for specific research areas, intensive government investment in stem cells didn't pick up until

2011, says Ma Zhun, an analyst with Lux Research in Shanghai. With calls for the Chinese biomedical materials industry to quintuple its share of the global market by 2050, such funding will almost certainly increase in decades to come.

Chinese tissue engineering research dates back to the 1990s, as the field was coming into its own internationally. Chinese scientists established a national Society of Tissue Engineering in 1999. That same year, they held the first national conference

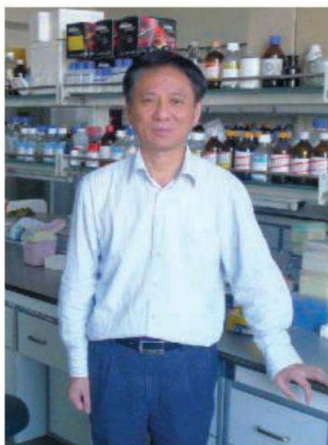
on the topic in Shanghai. But resources were limited. Myron Spector, a biomaterials scientist at the VA Boston Healthcare System and Brigham and Women's Hospital in Boston, recalls visiting Cui's lab in the late 1990s. Even at Tsinghua, one of China's top univer-

sities, researchers did not have sufficient support. “They didn't get journals, and when they did get them, they were old,” Spector says. He remembers returning to Boston and pricing out what it would cost to send boxes of the leading biomaterials journals to China.

Within a few short years, things changed. In 1997, plastic surgeon Cao Yilin, now one of China's leading tissue engineering researchers, made headlines when he was among a group of researchers in Boston to publish a paper in *Plastic and Reconstructive Surgery* relating his team's success in growing a human ear—comprised of tissue-engineered cartilage made from a biodegradable polymer seeded with chondrocytes—on a mouse. Cao was based in the United States at the time, working with prominent tissue engineer Joseph Vacanti at Massachusetts General Hospital, but he returned to China soon after. Others say his achievement helped prove the worth of the field to the Chinese government.

In 2001, a government grant program targeting research with commercial potential—the 863 Program—started explicitly funding tissue engineering. From 2002 to 2005, it supported an initial 11 projects in the field, according to Cui. The program currently sponsors 48 projects, says Guo Quanyi, who oversees the 863 Program's tissue engineering grants. The boom in funding has been matched by a steep increase in the number of professors and assistant professors in China working on tissue engineering, Cui says, from roughly 100 in the 1990s to some 300 today.

Cui was among those to benefit from the



Translational pioneer. Medical researcher Gu Xiaosong was early in taking artificial nerve grafts to the clinic.

Close at hand. Long tissue-engineered nerve grafts are being evaluated in clinical trials in China.

sudden onset of funding. When Spector revisited the Tsinghua professor's lab a few years later, he was shocked at the improvement in available resources and science being conducted. He listened in awe as Cui's graduate students described innovative biomaterials they were developing to treat defects in the brain resulting from conditions such as stroke. "I had just never heard anything like it," Spector recalls. "I thought, 'This is really out there.'"

Returning to the United States, he logged into the publication database PubMed and searched in vain for related research. He realized then that the Chinese scientists had pulled ahead of their Western counterparts in that particular subfield. "What happened in the West over many decades has been compressed into 2 decades" in China, Spector says. "There is definitely work in China that I would say is leading the rest of the world."

Cui himself is more circumspect about China's prospects. "Our lab conditions are comparable to those in the West. We are very good in some specific areas." But, he adds: "The overall research level in China is still behind the United States by 20 years." Others contend, however, that thanks to generous funding and a strong government emphasis on tissue engineering, the gap is closing very quickly.

Funding boom

Gu's lab exemplifies this progress. About 3% of trauma patients suffer from peripheral nerve damage that paralyzes them or impairs movement. Surgeons can repair such injuries by removing a nerve from elsewhere in the patient, from a leg, for example, and transplanting it at the site of the damaged nerve. But this approach entails two surgeries and the loss of one nerve segment in order to gain another. The alternative, artificial nerve grafts, can typically only bridge gaps of less than 30 millimeters, ruling out their use for more severe injuries.

So when Gu and colleagues began engineering nerve grafts that could bridge longer distances, the work attracted attention. His team was among the first in the world to develop nerve grafts using chitosan, a material usually derived from shrimp or crab shells, and the first to take such grafts to the clinic.

At the time, other teams around the world were working on chitosan-based nerve grafts. But Gu's method allowed scientists

to control the speed at which the chitosan conduit biodegrades—a quality "important for the repair of nerve defects with varying length, location, and diameter," says Yang Xiongli, a neuroscientist at Fudan University in Shanghai.

Gu was also the first to translate this artificial nerve research to the clinic. China's State Food and Drug Administration (SFDA) gave approval for clinical trials in 2010. A trial is now under way at four Chinese hospitals, with 35 grafts completed. Gu expects that it will conclude next year.

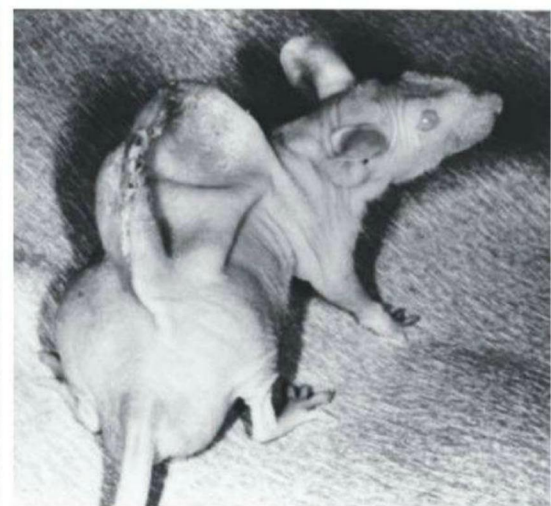
He's also combining his chitosan-based grafts with mesenchymal stem cells extracted



Mighty mouse. An artificial ear grown on a mouse by a U.S.-based team including Cao Yilin (fourth row, second from left) in the 1990s helped persuade China to invest in tissue engineering.

the Max Planck Institute of Neurobiology in Martinsried, Germany. And Gu has benefited from generous funding from China's two major grant programs, 863 and 973, as well as from the National Natural Science Foundation of China. The 973 Program focuses on basic research, and from 2009 to 2011, its funding for nanotechnology, a category that encompasses advanced materials, advanced diagnostics, electronics, and tissue engineering, totaled more than \$82 million, according to Lux Research. From 2006 to 2011, the 863 Program poured \$77 million into tissue engineering and stem cell research.

The sums given by the U.S. National Insti-



from bone marrow. In a study forthcoming in *Biomaterials*, Gu and colleagues describe how this approach bridged a 50-millimeter median nerve gap in rhesus monkeys. In addition, his team is developing other synthetic nerve grafts, including a material derived from a very Chinese substance: silk proteins. "The biocompatibility is better than with chitosan," Gu says.

Gu has pushed hard to make this progress. It hasn't hurt that for the past 3 years, he has served as Nantong University's Communist Party secretary, a powerful administrative post that ensures him a personal driver, access to education officials, and influence over key planning decisions, and that for the 4 years prior to that, he was the university's president. He is unabashed about steering resources toward nerve regeneration research: "Scientists who come here tend to end up doing neuroregeneration," he says.

Government largesse helped Gu construct his new lab, which he modeled after

tutes of Health and the Armed Forces Institute of Regenerative Medicine for tissue engineering research in the United States often exceed those awarded in China, says Liu Wei of the Shanghai Key Laboratory of Tissue Engineering, who cautions that it is difficult to compare funding in the two countries. But Chinese scientists often get matching funds from the local and provincial governments to sweeten the deal. The influx of cash means that China is positioned to "become one of the major players in this field," says James Yoo, a regenerative medicine specialist at the Wake Forest Institute for Regenerative Medicine in Winston-Salem, North Carolina.

Push toward translation

Gu's team is now trying to figure out why peripheral nerves can regenerate in people while other human nerves, such as the spinal cord, can't—a question that still perplexes researchers around the world. But such basic research questions may soon fall by the way-



Tissue engineering hot spots. Thanks to a boost in funding, China is home to research in a variety of areas, such as artificial skin and engineered organs.

side in China as more money becomes available for clinical research. “Five or 6 years ago it was enough for us simply to publish,” Gu says. “Now the government wants us to translate our research into clinical applications.”

China’s \$56 billion biomedical materials industry is largely driven by foreign technology; domestic companies accounted for a mere 3% of global market share in 2011. Government leaders have decided that’s too little, Ma says: “The current target is to commercialize tissue engineering technologies and bridge the gap between academia and industry.” Eyeing a lucrative market with potential for growth, the Chinese government recently set a goal of increasing China’s share of the global biomedical materials market to 10% to 15% by 2050.

As government interest in tissue engineering research shifts toward clinical translation, the funding split is changing as well. Tissue engineering “is a field with a huge capacity for innovation, and it promises solutions to a

lot of problems that modern medicine hasn’t been able to solve,” Guo says. He says the current batch of government-funded projects span cartilage, nerve, bone, heart, ligament, eye, and skin research.

Already, Gu has been particularly effective at applying his research, say scientists familiar with his work. “He understands the clinical needs,” says Yoo, whose institute signed an agreement earlier this year with the Jiangsu Key Laboratory of Neuroregeneration to establish a joint center.

And Gu is hardly alone. In 2007, SFDA approved China’s first tissue-engineered product: ActivSkin. Developed by researchers at the Fourth Military Medical University in Xi’an, it made China the second country in the world, after the United States, to possess artificial skin technology. Products approved since then include a bone repair scaffold developed by Cui that cleared SFDA in 2010. The material has been used by 30,000 patients in China and its application is being pursued elsewhere in the world, he says.

Translational research promises to become one of China’s strengths in tissue engineering, says Cao, the plastic surgeon whose work on cartilage engineering originally helped spark interest in the field. Now director of the Shanghai Key Laboratory of Tissue Engineering, he and Liu are working on clinical applications of engineered cartilage, bone, tendon, skin, and blood vessel grafts.

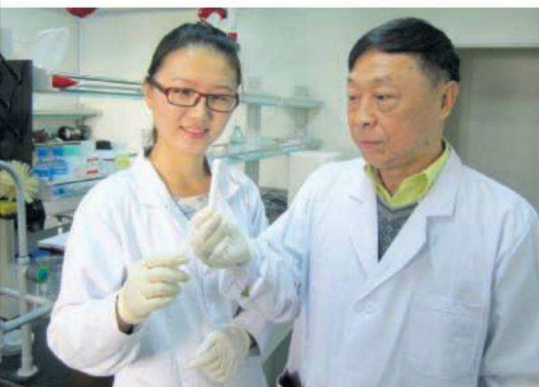
But the regulatory hurdles can still be substantial in China. Individual components of the grafting process require safety approvals before any clinical trials can start. Thus, Cao and Liu have yet to receive SFDA

approval to start clinical trials on any of their products. Moreover, SFDA staff members are reluctant to green-light trials for tissue-engineered devices that haven’t first been approved outside of China, Ma says. As a result, some Chinese scientists now apply for trials in Australia or the United States before seeking permission to work in patients domestically.

But the approval process for clinical trials can drag on for years. For many Chinese scientists, a lack of experience navigating their country’s regulatory agencies is an obstacle, says Dai Kerong, director of Shanghai Jiao Tong University’s Bone and Joint Research Center: “From bench to bedside—everybody knows it’s important, but most physicians don’t know how to do it.”

Bureaucratic intransigence may be only temporary. SFDA is investing money in developing more coherent regulatory standards, Ma says. And Dai is spearheading a series of seminars for tissue engineering scientists on how to navigate the trial application process and steer their research toward the clinic. “The important thing is China now has the funding mechanisms, both public and private, and the SFDA is now acting judiciously,” Spector says. But it could take time before more scientists achieve the success that Gu has had in moving their research into the clinic. “The central government has put a lot of money into this field,” says Dai, who mentored some of China’s younger tissue engineering researchers and remembers when things were very different. “The problem has become how to use it—so we don’t waste it.”

—MARA HVISTENDAHL



Bone doctor. Cui Fuzhai (right) developed a bone repair scaffold that has been used by 30,000 patients.

CREDITS (TOP TO BOTTOM): ADAPTED FROM INFORMATION COLLECTED BY LUX RESEARCH INC.; CUI FUZHAI

REVIEW

Multifunctional Nanoparticles: Cost Versus Benefit of Adding Targeting and Imaging Capabilities

Zhiliang Cheng,¹ Ajlan Al Zaki,¹ James Z. Hui,¹ Vladimir R. Muzykantov,² Andrew Tsourkas^{1*}

Nanoparticle-based drug delivery systems have been developed to improve the efficacy and reduce the systemic toxicity of a wide range of drugs. Although clinically approved nanoparticles have consistently shown value in reducing drug toxicity, their use has not always translated into improved clinical outcomes. This has led to the development of “multifunctional” nanoparticles, where additional capabilities like targeting and image contrast enhancement are added to the nanoparticles. However, additional functionality means additional synthetic steps and costs, more convoluted behavior and effects in vivo, and also greater regulatory hurdles. The trade-off between additional functionality and complexity is the subject of ongoing debate and the focus of this Review.

The continual identification and development of new drugs has led to an appreciable reduction in both mortality and morbidity for most life-threatening diseases, including cardiovascular diseases and cancer, the two leading causes of death in the world. However, because of poor pharmacokinetic profiles and broad mechanisms of action, most small-

molecule drugs still carry a substantial risk of systemic toxicity (1). Moreover, only a relatively small fraction of the administered drugs are delivered to and act at the disease site. Nanoparticles offer an opportunity to alter the pharmacokinetic profile of drugs, reduce off-target toxicity, and improve the therapeutic index. An example of such a formulation is Doxil (Janssen Biotech), a polyethylene glycosylated (PEGylated) liposome carrier loaded with the cytotoxic anticancer drug doxorubicin. Although doxorubicin indiscriminately diffuses across blood vessels into normal tissues and tumors alike, Doxil is generally retained within the blood pool except at sites of increased vascular permeability, such as in the liver and spleen and within tumors. As a

result, Doxil exhibits a circulation half-life that is 100 times longer than free doxorubicin and carries a risk of cardiotoxicity that is sevenfold lower than free drug (2). Yet, the use of Doxil does not always afford a significant improvement in survival compared with doxorubicin, for example, when used as first-line therapy in breast cancer patients (2).

The design and development of “multifunctional” nanoparticles seek to expand upon the benefits realized by Doxil and other first-generation, clinically tested nanoparticles (Table 1) by adding functionalities intended to improve delivery, therapeutic efficacy, and ultimately patient outcome. Multifunctional nanoparticles have been devised with stealthlike features to evade the immune system and prevent opsonization, protective layers to prevent the degradation of biologic cargo (e.g., proteins, DNA), targeting moieties to improve specificity and tumor accumulation, membrane-permeation moieties to improve cell uptake, imaging agents to assess delivery and dosing, endosome escape mechanisms, target-dependent assembly or disassembly to control drug release, microenvironment sensors (pH, proteases, phospholipases) to trigger drug release and cell uptake, and intracellular targeting moieties to direct drugs to specific intracellular compartments. Some of these features are considered necessary for the delivery of therapeutic biologics, such as peptides, proteins, small interfering RNA (siRNA), and genes; however, the cost/benefit ratio of these modifications in improving the delivery of many small-molecule drugs is less certain. Each new functionality elevates complexity (e.g., multistep syntheses, purification, and characterization) and cost (e.g., lower yields,

¹Department of Bioengineering, University of Pennsylvania, 240 Skirkanich Hall, 210 South 33rd Street, Philadelphia, PA 19104, USA. ²Institute for Translational Medicine and Therapeutics and Department of Pharmacology, University of Pennsylvania School of Medicine, TRC 10-125, 3400 Civic Center Boulevard, Building 421, Philadelphia, PA 19104, USA.

*To whom correspondence should be addressed. E-mail: atsourk@seas.upenn.edu

Table 1. Nontargeted nanoparticles that have been approved for clinical use or undergoing clinical trials. PLA, poly(L-lactide); pAsp, poly(L-aspartic acid); PGlu, polyglutamate; PAA, poly(L-aspartate); HPMA, N-(2-hydroxypropyl)-methacrylamide copolymer.

Brand name	Composition	Indication	Status
<i>Liposome-based nanoparticle</i>			
Doxil/Caelyx	PEGylated liposomal doxorubicin	Ovarian cancer, Kaposi's sarcoma	Approved
DaunoXome (Galen)	Liposomal daunorubicin	Kaposi's sarcoma	Approved
Myocet (Sopherion)	Non-PEGylated liposomal doxorubicin	Breast cancer	Approved
<i>Micelle-based nanoparticle</i>			
Genexol-PM	Paclitaxel-loaded PEG-PLA micelle	Breast cancer, lung cancer	Approved
NK911	Doxorubicin-loaded PEG-pAsp micelle	Various cancers	Phase 2
NK012	SN-38-loaded PEG-PGlu(SN-38) micelle	Breast cancer	Phase 2
NC-6004	Cisplatin-loaded PEG-PGlu micelle	Various cancers	Phase 1
SP1049C	Doxorubicin-loaded pluronic micelle	Gastric cancer	Phase 3
NK105	Paclitaxel-loaded PEG-PAA micelle	Breast cancer	Phase 3
<i>Polymer-drug conjugates-based nanoparticle</i>			
OPAXIO (Cell Therapeutics)	Paclitaxel combined with a polyglutamate polymer	Ovarian cancer	Phase 3
IT-101	Camptothecin conjugated to cyclodextrin-based polymer	Various cancers	Phase 1/2
HPMA-DOX (PK1)	Doxorubicin bound to HPMA	Lung cancer, breast cancer	Phase 2
HPMA-DOX-galactosamine (PK2)	Doxorubicin linked to HPMA bearing galactosamine	Hepatocellular carcinoma	Phase 1/2
CT-2106	Camptothecin poly-L-glutamate conjugate	Various cancers	Phase 1/2
<i>Albumin-based nanoparticle</i>			
Abraxane	Albumin-bound paclitaxel nanoparticles	Metastatic breast cancer	Approved

more costly materials), and regulatory barriers arise (e.g., owing to multicomponent, heterogeneous formulations). This Review focuses on the benefits and caveats of adding targeting and imaging features to nanoparticles as a means to improve the efficacy of small-molecule anticancer drugs (Fig. 1), because these modifications are expected to see the most immediate translation into the clinic. In fact, several targeted nanoparticles have already shown sufficient promise for entry into clinical trials (Table 2).

Targeting: The Good, the Bad, and Cell Uptake

The accumulation of nontargeted nanoparticles, which includes all current Food and Drug Administration (FDA)-approved nanoparticles, within malignant lesions is generally attributed to passive (pathophysiological) targeting. Passive

targeting is a consequence of enhanced permeability and retention (EPR), whereby the leakiness of the tumor vasculature combined with poor lymphatic drainage enable nanoparticles to accumulate within the tumor matrix. Animal studies suggest that EPR can lead to more than a 50-fold increase in nanoparticle accumulation within tumors compared with healthy tissues (3). In general, the longer the nanoparticle circulation time, the greater the EPR-induced accumulation (4).

Although there is no single optimal particle size or surface charge for maximizing EPR because of the high degree of tumor-to-tumor variability, nanoparticles between 10 and 100 nm typically demonstrate the most effective tumor penetration (5), but particles up to ~400 nm in size have been shown to extravasate into tumors in animal models (6). However, the EPR effect is

not commonly observed in some types of cancers, including gastric and pancreatic cancer (7). In other cases, the tumor core may not be well perfused (8).

Over the years, there have been a number of adjunct strategies that have been shown to increase vascular permeability, which may be used to enhance nanoparticle accumulation at the tumor site. One such approach involves the administration of vasoactive agents such as vascular endothelial growth factor, thrombin, bradykinin, substance P, and lipopolysaccharide endotoxin. These agents initiate a cascade of cellular events that ultimately result in disruption of endothelial cellular junctions and increase vascular permeability. An alternative method for increasing vascular permeability includes transiently raising systemic pressure by infusing vasoconstrictors

(e.g., angiotensin II) to overcome the high interstitial pressure of tumors (9). One general concern with all of these methods is that altering blood vessel permeability may affect both healthy blood vessels and diseased blood vessels and therefore may increase the occurrence of off-target effects. More targeted approaches involve the use of external stimuli such as ionizing radiation to alter vascular permeability (10) or photodynamic therapy to disrupt vascular integrity (11). It has been suggested that systemic injection of the tumor-penetrating peptide iRGD may also increase vascular and tissue permeability in a tumor-specific manner, through interactions with integrin and neuropilin-1 (12).

Presumed benefits of developing receptor-specific targeted nanoparticles. Active targeting mediated by affinity ligands may complement EPR or provide an alternative delivery mechanism. The value of targeting therapeutic agents can be seen from early clinical trials with antibody-conjugated chemotherapeutics. T-DM1, which consists of the antibody trastuzumab (Herceptin, Genentech) linked to the cytotoxin mertansine, was shown to reduce the death rate of patients with human epidermal growth factor receptor 2 (HER2)/neu-positive metastatic breast cancer by 37% and side effects by nearly 50% in phase III clinical trials (13). The selective toxicity of this and similar agents is attributed to an increase in the target/nontarget distribution ratio. Similar results have also been reported in many animal studies with targeted nanoparticles. For example, in a murine tumor model, folate-targeted paclitaxel-loaded micelles exhibited a tumor accumulation of $10 \pm 2\%$ injected dose per gram of tissue (% ID/g) compared with only $1 \pm 0.3\%$ ID/g for nontargeted micelles and a markedly reduced systemic toxicity (14). Nanoparticles offer an opportunity to carry a larger and more

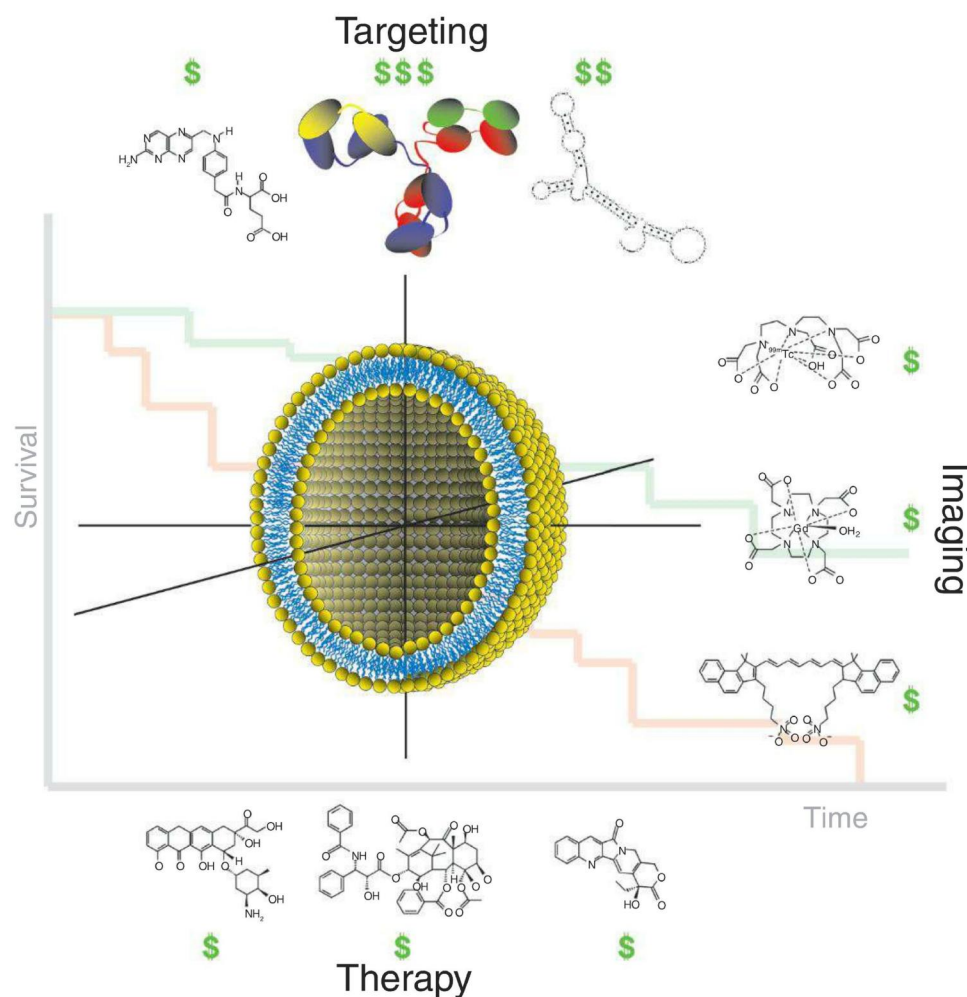


Fig. 1. Schematic of a multifunctional nanoparticle. Multifunctional nanoparticles can be prepared with a wide range of therapeutic, imaging, and targeting agents. Representative examples are shown, with relative expenses indicated by dollar symbols. The addition of each new functionality is expected to have a beneficial impact on patient survival; however, this will come at the cost of additional regulatory, production, and financial hurdles. The trade-off between these “costs” and clinical benefit will be highly dependent on the choices made when designing multifunctional nanoparticles. The following agents are shown: Targeting (left, folic acid; center, antibody; right, aptamer); Imaging (top, chelated Technetium-99m; center, chelated gadolinium; bottom, near-infrared fluorescent dye isocyanine green); Therapy (left, doxorubicin; center, paclitaxel; right, camptothecin).

Table 2. Targeted nanoparticles in clinical development for use in cancer therapy.

Name	Composition	Target	Comments	Status
MCC-465	PEGylated liposomal doxorubicin containing antibody GAH targeting agent	Tumor antigen	Metastatic stomach cancer	Phase 1
MBP-426	Liposomal oxaliplatin containing human transferrin protein targeting agent	Transferrin receptor	Advanced or metastatic solid tumors	Phase 1/2
SGT-53	Liposomal p53 cDNA containing antibody fragment targeting agent	Transferrin receptor	Solid tumors	Phase 1
CALAA-01	siRNA-loaded polymeric nanoparticles containing human transferring protein targeting agent	Transferrin receptor	Solid tumors	Phase 1
BIND-014	Docetaxel-loaded polymeric nanoparticles containing peptide targeting agent	Prostate specific antigen	Advanced or metastatic cancer	Phase 1

diversified drug payload than direct antibody conjugates, including poorly water-soluble drugs, which can account for ~40% of the active substances identified through combinatorial screening (15).

A selective increase in the delivery of small-molecule drugs to cancer cells requires the functionalization of nanoparticles with targeting ligands that bind to cognate counterparts preferentially expressed on the target cells (Table 3). Targeting can be particularly valuable in treating small metastases (<100 mm³), because these sites are poorly vascularized and do not evoke EPR (16). Accordingly, in a murine orthotopic model for pancreatic carcinoma, $\alpha_v\beta_3$ -targeted doxorubicin-loaded nanoparticles led to an 82% reduction in the growth of metastatic lesions compared with untreated control animals. In contrast, nontargeted nanoparticles did not show any significant effect on metastatic lesions (17). Furthermore, it has been shown that targeted nanoparticles are able to overcome multiple drug resistance because glycoprotein efflux pumps are unable to remove drug-nanoparticle complexes that have entered via receptor-mediated events (18).

Drug-loaded nanoparticles may also exhibit a synergistic tumoricidal effect when actively targeted with therapeutic antibodies, that is, combining chemotherapy and immunotherapy. For example, when doxorubicin-loaded nanoparticles were functionalized with the therapeutic antibody trastuzumab, they exhibited more effective inhibition of tumor growth compared with the additive effect of therapeutic antibodies and nontargeted doxorubicin-loaded nanoparticles when each was administered individually (19).

As an alternative to targeting tumor cells, other options include targeting the endothelium, to choke the blood supply and starve the cancer cells of nutrients and oxygen, and targeting tumor-supporting cells such as tumor-associated macrophages. When DNA vaccines were targeted to legumain, a stress protein that is overexpressed on tumor-associated macrophages, mouse models of metastatic breast, colon, and non-small cell lung cancer displayed prolonged survival in 75% of mice, and 62% were completely free of metastasis (20).

Challenges facing the development of receptor-specific targeted nanoparticles. It has been more than 30 years since the concept of targeted nanoparticles was introduced (21), but few formulations have reached clinical trials and to date none have been clinically approved (Table 2). Although targeted therapeutics hold much promise, there are also potential risks and challenges, ranging from synthesis and purification, choosing the right ligand-receptor pair, to altering nanoparticle properties. Even the conjugation technique can affect binding features of a ligand because of conformational changes, insufficient steric freedom, or inadequate orientation. Promising novel bioconjugation methods including click chemistry are being introduced to overcome some of these limitations, but their adaptability, biological inertness, and clinical potential remain to be thoroughly appraised (22).

In addition to practical constraints, some have questioned whether targeting actually improves nanoparticle accumulation in tumors. A fundamental paradox here is that the addition of targeting moieties compromises the stealth feature of nanoparticles and can accelerate their clearance by the host. For example, it has been shown that nontargeted liposomes can exert comparable tumor accumulation as folic acid-functionalized liposomes because they benefit from longer circulation times and higher EPR (23). The density of the target receptor on tumor cells may also be a limiting factor. In a metastatic breast cancer model, it was found that a receptor density of 10⁵ HER2/neu receptors per cell was required to achieve an improved therapeutic effect with anti-HER2/neu-targeted liposomal doxorubicin over nontargeted liposomal doxorubicin (24).

Not all cancer cell types overexpress the same unique receptors, and often overexpressed receptors are also present on normal tissue. A recent study of gastric cancer found that significant intratumoral heterogeneity also exists, with 79.3% of HER2-positive tumors having regions with different immunohistochemical staining scores (25). Some cancer cells, for example, cancer stem cells, may be void of any known up-regulated recep-

tors (26). The dynamic nature of tumor markers is also a challenge. In an animal model, it was found that Sigma receptor density decreased by 30% after a single dose of doxorubicin (27) and Her2/neu receptor density decreased by 40% after treatment with trastuzumab-conjugated nanoparticles (28). In this context, nanoparticles with multiple unique targeting ligands have generally been seen as an advantage that may help to defuse this problem. Testing of this “multispecificity” approach is ongoing (29).

Nanoparticle multivalency and multispecificity can also help to overcome the weak affinity of some ligand-antigen pairs and promote endocytic uptake. Hong *et al.* showed that a fivefold increase in the number of folic acid on dendrimer nanoparticles gives a 68-fold improvement in binding avidity (30). However, multivalency can increase avidity and cause unexpected adverse cellular responses. Wang *et al.* showed that transferrin and transferrin receptor antibodies, neither of which are toxic individually, can be toxic to selected cells when multivalently conjugated to nanoparticles (31). Although multivalency-induced toxicity can be leveraged against cancer cells, further investigation into the optimal level of multivalency and related adverse effects for each nanoparticle formulation is nonetheless warranted (32). Further, an increase in valency does not necessarily translate into more effective targeting. In some cases, nanoparticles with an intermediate ligand density display higher binding than nanoparticles with higher ligand densities, likely because of steric limitations imposed on congruency intermolecular ligand-receptor interactions by too closely adjacent ligands (33).

Although higher nanoparticle avidity is generally seen as an advantage, the use of targeted nanoparticles with high avidity may elicit a “binding site barrier” wherein binding to target cells paradoxically reduces penetration in deep layers of the tumors (34). This was originally observed with antibodies and may be particularly problematic for nanoparticles because of higher diffusion limitations. Because the binding site barrier is more prominent at low doses, this may negate

Table 3. Common tumor biomarkers that can potentially be used as nano-particle targets. PSMA, prostate-specific membrane antigen; PCLA, prostate cancer lipid antigen; MUC1, mucin-1; VCAM-1, vascular cell adhesion molecule 1; VEGFR, vascular endothelial growth factor receptor; Tem1, tumor endothelial marker 1; TAMs, tumor-associated macrophages; TAFs-FAP, tumor-associated fibroblasts–fibroblast activation protein; TEMs, Tie-2–expressing monocytes; APA, aminopeptidase A.

Tumor biomarker	Expression in cancer tissues (% of tumors that express biomarker)	Expression in normal tissues/cells	Ref.
<i>Solid tumors</i>			
Folate receptor	Ovarian cancer (90%), renal cancer (86%), lung cancer (72%), breast cancer (43%), brain cancer (25%), pancreatic cancer (50%)	Kidney, colon, lungs, placenta, bladder	(75)
EGFR	Non–small-cell lung cancer (40–80%), colorectal cancer (50–80%), ovarian cancer (35–70%), gastric cancer (41–83%), pancreatic cancer (30–50%), breast cancer (14–91%), bladder cancer (31–72%), head and neck (80–100%), glioma (40–63%)	Cells that originate from all three germ cell layers, particularly those of epithelial origin (e.g., the skin, liver, and gastrointestinal tract)	(76, 77)
HER2	Non–small-cell lung cancer (18–37%), colorectal (26–90%), ovarian cancer (10–15%), gastric (38–45%), breast cancer (25–30%), bladder cancer (9–36%), glioma (20–54%)	Skin, breast, placenta, epithelial cells on gastrointestinal, respiratory, reproductive and urinary tract	(76, 78)
PSMA	Prostate cancer (56.7–100%), high-grade prostatic intraepithelial neoplasia (48.6–100%)	Prostate, kidney, small bowel, colon	(79, 80)
PCLA	Prostate primary tumor (96.6%), metastatic prostate carcinoma (85.3%)	Brain vasculature, benign prostate tissue	(81)
Transferrin receptor	Colon cancer (48%), breast cancer, kidney cancer, lung cancer, stomach cancer, ovarian cancer*	Skin, pancreas, liver, brain (anterior pituitary), testis	(82, 83)
MUC1	Breast cancer (90%), lung cancer, prostate cancer and colorectal cancer*	Mammary gland, respiratory, urinary and reproductive tracts	(84, 85)
<i>Tumor vasculature</i>			
$\alpha_v\beta_3$ integrins	Melanoma, breast cancer, prostate cancer, pancreatic cancer, ovarian cancer, cervical cancer, glioblastoma, and tumor endothelial vessels*	Platelets, very low levels in resting endothelial cells and normal organs	(86)
VCAM-1	Leukemia, lung and breast cancer, melanoma, renal cell carcinoma, gastric cancer*	Up-regulated on endothelial cells in response to inflammation	(87)
VEGFR	Highly expressed on neovascular endothelial cells**, 73%–100% in some non-small cell lung cancers	Monocytes, macrophages	(88)
Tem1	Highly expressed on neovascular endothelial cells** including colon, brain and lung cancers	Expressed on normal endothelial cells	(89, 90)
APA	Up-regulated on perivascular cells of tumor blood vessels, Stromal cells surrounding prostatic carcinoma cells (73%), nonkeratinizing type cervical squamous cell cancer (90%) renal cancer (clear cell cancer)*	Expressed in the proximal tubules and glomerulus of nephron (kidney), up-regulated in inflamed synovia, granulation tissue, low expression in capillaries and venules of pancreas, lymphoid tissue and intestinal mucosa	(91–94)
<i>Supporting cells</i>			
TAMs	Breast, prostate, ovary, cervix, stomach, lung, glioma, and bladder cancers*	**	(95)
TAFs–FAP	Overexpressed in 90% of stromal fibroblasts in colon, lung, and breast carcinoma	Fibroblasts in healing and inflammation	(96)
TEMs	*	Endothelial cells, hematopoietic cells	(97)

*Percentages of tumors expressing these receptors is not available. **Not applicable.

the perceived advantage of administering lower dosages with targeted nanoparticles. Lastly, high avidity may compromise selectivity, because nanoparticles may be depleted upon binding to non-tumor cells expressing low levels of “tumor-specific determinants” (35). An absolute specificity of a target molecule for cancer cells (or any other targets) is unlikely to exist. In fact, many “tumor-specific” target molecules, including receptors for folate, integrins, and transferrin, exist in nontumor cells and pose the danger of off-target (yet specific) binding and effects (Table 3).

These considerations help to explain a co-existence of reports of increased tumor accumulation of targeted nanoparticles with those showing increased cellular internalization but not tumor accumulation. The view that targeting does not necessarily increase localization is supported by theoretical works, which suggest that nanoparticles reach cancer cells by enhanced permeability (36). Ligands then bind target receptors, which helps to retain the nanoparticles within the tumor. However, nanoparticles must still overcome high intratumoral fluid pressure and penetrate the dense tumor extracellular matrix (37, 38). Adding a targeting moiety may hamper this process by increasing the nanoparticle size (which further impedes diffusion) (39) and creating the affinity barrier problem discussed above. These sobering considerations on the real value of targeting pertain mostly to the targeting of cancer cells in primary solid tumors. Tumor penetration is much less relevant in situations where the tumor endothelium or other readily accessible targets are pursued. Recent findings suggest that coordinated targeting approaches may provide a solution to poor tumor penetration. It has been shown that iRGD can specifically home to tumors and promote tissue extravasation through coordinated interactions with both integrin and neuropilin-1 (40).

Cellular internalization. If targeting does not help nanoparticles actively reach the tumor, then the true value of targeted nanoparticles may lie in their ability to be internalized upon receptor-mediated endocytic processes. Cell internalization is considered to be important because many of the FDA-approved nanoparticle-based medications, such as Abraxane (paclitaxel, Celgene) and Doxil (doxorubicin), contain drugs that act on intracellular targets. Therefore, internalization is expected to result in higher intracellular drug concentrations and facilitate drug-target interactions. Commonly used targeting ligands, such as transferrin, epidermal growth factor (EGF), and many antibodies, already benefit from receptor-mediated endocytic processes. Even ligands that do not naturally internalize often undergo internalization when displayed multivalently on the nanoparticle surface (41).

Targeting to specific epitopes may also help to control the nanoparticle's intracellular trafficking and destination. The transferrin receptor, which recycles to the plasmalemma and thus avoids lysosomal degradation (31) and glycoproteins, localizes in caveoli, favoring transcellular transport (42). Dovetailing on such natural transport mechanisms may assist in subcellular delivery of vulnerable biotherapeutics. However, the uptake and trafficking of ligand-guided nanoparticles may drastically differ from those of ligands in their natural form. Bhattacharyya *et al.* showed that using nanoparticles conjugated with cetuximab [an antibody against EGF receptor (EGFR)], compared with free cetuximab alone,

uniformly enhanced EGFR endocytosis, changed the compartmentalization pattern, and implicated the role of a distinct pathway involving dynamin-2 in the endocytic processes (43).

The use of high-affinity ligands may also impede physiological mechanisms, including nanoparticle detachment from the receptor and intracellular transport. For example, low-affinity antibody conjugates targeted to transferrin receptor provide more effective transport across the vascular barrier by virtue of more dynamic interaction with the receptor than high-affinity counterparts (and likely because of an elevation in the ratio of free/bound conjugate in the vasculature) (18). Parameters of nanoparticle design, such as size, shape, valency, and plasticity, greatly modulate subcellular trafficking (44) and need to be more fully understood in order to capitalize on the potential advantages provided by targeting.

New targeting strategies that exploit the tumor microenvironment or external stimuli. Targeting cell-surface biomarkers is complicated and mostly empiric. New targeting strategies that exploit the altered tumor microenvironment may avoid many of the complications and pitfalls associated with receptor-specific targeting and achieve broader disease coverage (Fig. 2). For example, targeting agents have recently been designed to use local factors of the tumor microenvironment to trigger cell uptake. In one approach, nanoparticles were linked to neutralized cell-penetrating peptides that become activated upon cleavage by the metalloproteinases present in the tumor parenchyma, enabling local intracellular delivery of the cargo (45).

A plethora of pH-sensitive drug-release, cell-penetrating, and disassembly mechanisms have also been devised to assist tumor targeting by taking advantage of the lower pH observed in most tumors. Metabolic differences that exist between tumors and normal tissues have been known for a long time: Increased glucose uptake and glycolysis lead to tumor acidification, a phenomenon known as the Warburg effect. Recent examples of nanoparticles that target the acidic tumor microenvironment include nanoparticles functionalized with glycol chitosan, a water-soluble polymer with a pH-titrable charge (46). These metabolic sensors undergo a switch from a near-neutral charge at pH = 7.4 to a positive charge at lower pH, enabling them to remain in the acidic tumor microenvironment because of electrostatic interactions with surrounding tissue. In another approach, pH low-insertion peptides have been developed that are soluble in physiologic pH but form a rigid transmembrane α -helix that spontaneously inserts across lipid bilayers at low pH (<7.0) (47). It has been shown that these insertion peptides can be used to translocate nanoparticles across lipid membranes (48). Preliminary studies have suggested that these peptides can insert into primary tumors, metastatic lesions, and lipid

bodies in necrotic tissues (49). Environment-responsive liposomes have also been prepared by anchoring phospholipids with cell-penetrating peptides and a pH-sensitive PEG shield. At low pH, the liposomes lose their PEG coating and are internalized into cells via the now-exposed cell-penetrating peptide moieties (50). In a similar fashion, nanoparticles have also been designed to shed their PEG coating in response to other stimuli, including reducing agents and proteases, exposing targeting ligands or positive charges (51). Although PEGylation is required to impart stealthlike properties on nanoparticles, it also limits drug release; therefore, shedding of the PEG coating can facilitate drug delivery. Overall, it is expected that these microenvironment-sensitive delivery approaches may provide a new mechanism for the treatment of tumors. On a cautionary note, tissue acidosis is not a tumor-specific phenomenon; areas of pathological (ischemia) and physiological (e.g., skeletal muscle in exertion) acidification may become victims of off-target localization of pH-regulated delivery systems.

As an alternative to relying on environmental stimuli to drive nanoparticle localization, some nanoparticle formulations can be externally manipulated. It has been shown that iron oxide nanoparticles with bound anticancer agents can selectively accumulate in cancer tissues under the guidance of a magnetic field gradient. Researchers found that, by using magnetic drug targeting of ferrofluids, only 20% of the systemic dose was necessary for complete remission of tumors, and no side effects were observed (52). This approach mirrors other targeted approaches in which external stimuli are applied to limit treatment to the tumor site, such as photodynamic therapy (53), nanoparticle-mediated hyperthermal therapy (54), and photoactivatable nanoparticles that release their cargo in response to light (55).

Combining Drugs with Imaging Agents: Synergistic or Counterproductive?

Benefits of developing theranostic nanoparticles.

There has been a trend toward combining therapeutic and diagnostic functions within a single formulation at the nanoscale, that is, "theranostic" agents. Compared with delivering drugs or imaging agents alone, theranostic agents can simultaneously deliver imaging and therapeutic agents to specific sites or organs, enabling detection and treatment of disease in a single procedure. When drugs and imaging agents are combined into a single formulation, they are usually assumed to have similar biodistribution and tumor localization in living subjects. As a result, theranostics agents are expected to inform us about the localization of the drug and pathological process longitudinally. Direct visualization of theranostic pharmacokinetics can provide important insight into heterogeneities between tumors and patients. This will help physicians make informed decisions about timing, dosage, drug choice, and

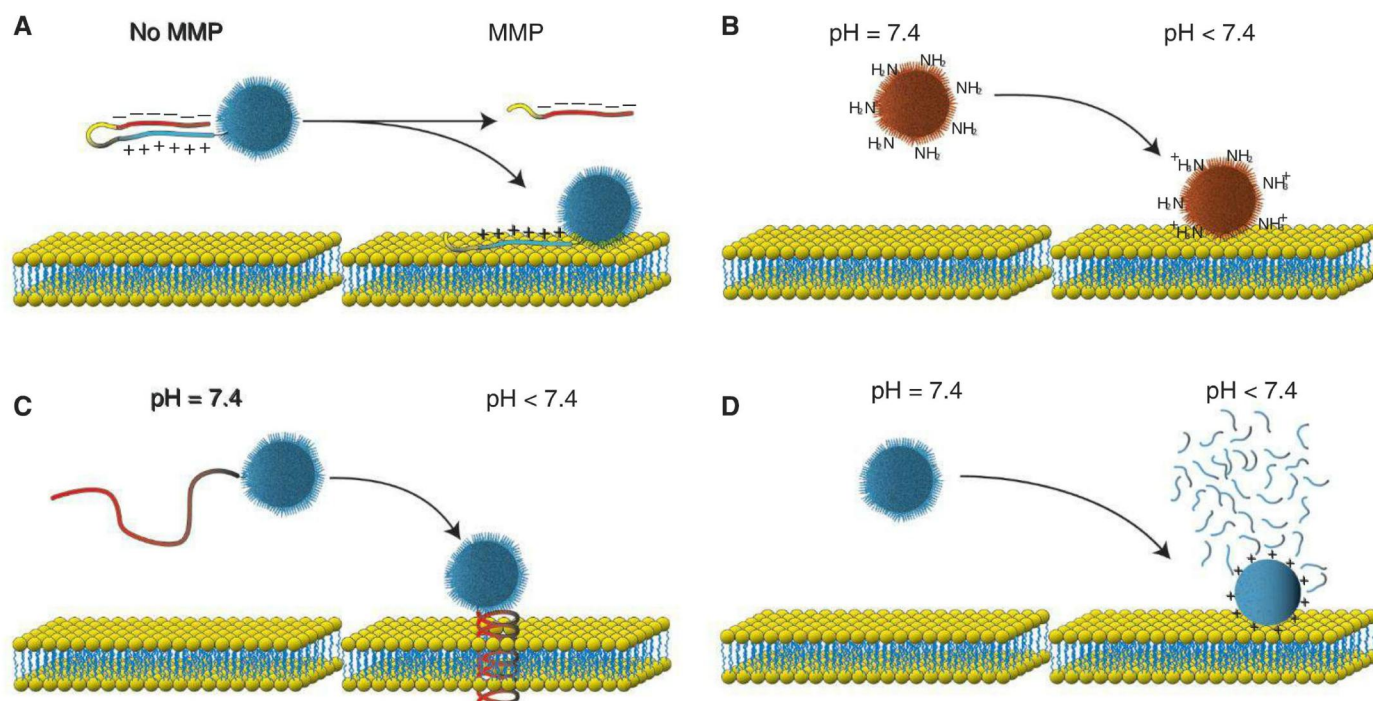


Fig. 2. Schematic diagrams of targeted nanoparticles that use local factors from the tumor microenvironment to improve drug delivery to tumors. **(A)** Carriers containing matrix metalloproteinase (MMP)–sensitive cell-penetrating peptides. A polycationic cell penetrating peptide is connected to a neutralizing polyanion via a MMP-cleavable linker. Dissociation of the polyanion after MMP-mediated cleavage enables the activated cell-penetrating peptide to associate with and enter cells. **(B)** pH-responsive glycol chitosan (GC)–nanoparticles. At physiologic pH, the surface amines on the GC-nanoparticles are deprotonated and carry a net neutral charge. Within the acidic tumor microenvironment, the GC-nanoparticles acquire a net positive surface charge

and exhibit electrostatic interactions with negatively charged cell membranes, leading to enhanced tumor retention. **(C)** Action of pH low insertion peptide (pHLIP). pHLIP is a water-soluble membrane peptide that shows little to no interaction with cell membranes at physiologic pH, but at slightly acidic pH forms a stable transmembrane α -helix and inserts into the cell membrane. **(D)** pH-responsive nanoparticles. Nanoparticles shed their protective coating within an acidic tumor microenvironment, exposing an underlying positive surface charge that promotes electrostatic interactions with surrounding cells. This “unmasking” process may also be triggered by other stimuli, including reducing agents and proteases.

treatment strategies. Such “personalized medicine” can lead to improved efficacy, lower off-target toxicity, and an overall increase in quality of life and patient outcome.

In an example illustrating these aspirations (56), a theranostic agent was prepared by the encapsulation of iron oxide nanoparticles and the photosensitizer Photofrin (Pinnacle Biologics) within polyacrylamide nanoparticles. Dynamic scanning magnetic resonance imaging (MRI) revealed that tumor-targeting resulted in an increase in tumor half-life from 39 to 123 min, within a rat brain tumor model. The therapeutic efficacy of nanoparticles was subsequently evaluated in comparison to the free photosensitizer and was monitored by changes in tumoral diffusion, as measured by MRI. Treatment of glioma-bearing rats with targeted Photofrin-encapsulated nanoparticles resulted in a higher mean tumor apparent diffusion coefficient compared with those of nontargeted control nanoparticles and free Photofrin.

Challenges facing the use of theranostic nanoparticles. In many cases, the addition of a contrast agent to a drug carrier might enable some imaging capabilities but will still be below the threshold for obtaining images with sufficient

quality or resolution. For example, micelles and liposomes that have their outer surfaces labeled with chelated gadolinium (Gd) will typically only possess several hundred to several thousand Gd per nanoparticle (57). In contrast, state-of-the-art Gd-based MR contrast agents can have tens to hundreds of thousand Gd per particle (58–60). Similarly, loading nanoparticle-based imaging agents with anticancer drugs is not necessarily a winning proposition. For example, labeling the surface of superparamagnetic iron oxide nanoparticles with small molecule drugs such as doxorubicin would only allow for the incorporation of several hundred to several thousand doxorubicin molecules per particle (<4% w/w) (61), far below some of the state-of-the-art drug delivery vehicles, which can have drug loading efficiencies of 30 to 70% w/w (62–66).

The design of theranostic agents can require a compromise between optimal features of sole modalities. Therefore, the question is how much imaging and therapeutic efficacy one is willing to sacrifice in order to achieve the benefits of having dual functionality. This will likely depend on how much value the imaging agent can provide and whether it will affect patient man-

agement. In general, the combination of imaging and therapeutic agents is not a natural fit. For example, although long circulation times are generally preferred for therapeutic nanoparticles to maximize total tumor accumulation via EPR, this is not ideal for imaging. Imaging can only be conducted once sufficient contrast agent has been cleared from circulation; otherwise, the background signal may be too high to accurately quantify tissue biodistribution. Moreover, nanoparticles can often have long residence time at the tumor site, particularly those possessing inorganic materials. This may limit the ability of nanoparticle-associated imaging agents to provide temporal information on changes in biomarker expression, tumor response, and vascular reorganization. Consequently, adding imaging agents to therapeutic nanoparticles may simply serve as a way to track nanoparticle biodistribution.

The addition of imaging capabilities to therapeutic nanoparticles increases the cost and the complexity of synthesis and purification and will lead to more heterogeneous formulations. Therefore, it must be carefully considered whether or not individualized information on nanoparticle distribution warrants the additional expense. With

a dedicated imaging agent, the contrast enhancement would likely be superior to theranostic agents, images can be acquired at more convenient times and intervals, and targets can be chosen that are independent of the therapeutic target to better reflect tumor response, with less concern about altered expression owing to drug resistance. Perhaps an optimal resolution is to adopt two mutually exclusive imaging agents (e.g., different energies or modalities) that will allow for the parallel tracking of nanoparticle biodistribution and monitoring of tumor response. Some imaging agents may also be able to be temporally resolved, owing to distinct radionuclide decay and/or clearance profiles.

The immediate future of theranostic nanoparticles. Theranostic agents are being devised with diverse contrast media, including those for optical imaging. Arguably, isotope probes enabling single-photon emission computed tomography (SPECT) and positron-emission tomography (PET) offer preferable features for clinical translation: high sensitivity, accurate quantification, deep tissue penetration, and real-time noninvasive longitudinal imaging. Labeling nanoparticles with isotopes enables the accurate assessment of biodistribution, circulation half-life, and pharmacokinetics of nanoparticles. Because of the high sensitivity of nuclear imaging and well-controlled labeling, patients can be treated with carriers loaded with high therapeutic doses yet labeled with rather trace amounts of isotopes that do not compromise drug loading and safety. Nonetheless, when combining radioisotopes with therapeutic nanoparticles, there are several issues that must be taken into consideration.

The short half-life of most clinically suitable radionuclides has generally led to their placement on the surface of therapeutic nanoparticles to facilitate expedited labeling and purification. When using nanoparticles with surface-bound radionuclides, it is important to ensure stability of the radiolabel. For example, if a radiometal is lost from its chelate or if the radiochelate is removed prematurely from the nanoparticle, acquired images would not accurately reflect actual nanoparticle biodistribution and could lead to misleading findings. Studies suggest that the *in vivo* behavior of metal chelates exposed to a complex protein environment cannot be predicted by classical equilibrium constants, so *in vivo* evaluation is often warranted (67). Approaches for stable radiolabeling of nanoparticles are needed; however, recent animal studies using nanoparticles stably labeled with PET isotopes show promising results (68).

To close the gap between the desired long circulation time for therapeutic nanoparticles and the short time frame desired for nuclear imaging, devise of targeted theranostics seems an interesting avenue. Although targeting may not always lead to elevation of tumor accumulation, it may accelerate this process, which is advan-

tageous for imaging. For example, whole-body gamma scintigraphic imaging of mice revealed noticeable tumor contrast 6 hours postinjection when administering targeted ^{111}In -radiolabeled liposomes, compared with 24 hours for nontargeted nanoparticles (69).

As an alternative to radiolabeled nanoparticles, nanomaterials exhibiting an intrinsic utility for both imaging and therapy seem ideal for theranostic agents. For example, iron oxide nanoparticles can be used for both MR imaging and thermal ablation therapy (70), gold nanoparticles for computed tomography (CT) imaging and radiation therapy (71), gold nanoparticles for CT imaging and thermal ablation therapy (71), and porphyrins for photoacoustic imaging and photodynamic therapy (72). When irradiated with the appropriate form of energy, such as near infrared light, radiowaves, or an alternating magnetic field; porphyrins, iron oxide, and gold nanoparticles absorb the incident energy and convert it to heat, which kills the cancerous cells. In the case of radiation therapy, the presence of gold results in radiation dose enhancement primarily owing to a photoelectric effect.

Although still in a relatively early stage of technological development, these theranostic agents show promise for cancer therapy because of their simplicity, cost effectiveness, and their ability to treat chemotherapy-resistant cancers. However, it should be noted that the delivery of high doses of these agents to tumors are often needed, and in many cases a direct intratumoral injection is required.

Perspective

Multifunctional nanoparticles featuring targeting ligands and other auxiliary moieties remain to be translated into the clinical domain, and the discussion of their utility and cost/benefit ratio seems timely. At the moment, the conventional point of view is that adding targeting ligands to therapeutic nanoparticles is worth the additional complexity of synthesis, cost, and regulatory hurdles. The targeted nanoparticles that minimize these issues are likely to see the most immediate clinical translation. Nanoparticles that do not require postsynthesis modification and purification may provide one such solution. This can be accomplished by coupling low-molecular-weight targeting ligands (e.g., small molecules like folate, peptides, and aptamers) or bioresponsive materials (e.g., pH-responsive polymers) directly onto nanoparticle starting materials before nanoparticle formation. This allows tighter control over synthesis, uniformity, and target ligand density. Although natural biological proteins, e.g., transferrin, require postsynthesis conjugation and purification, their abundance and relatively low cost also make them a favorable option. Nearly all of the targeted nanotherapeutics that are in early clinical trials use transferrin as a targeting ligand (Table 2). Of course,

the choice of natural ligands is very limited. Antibodies present the most flexible and perhaps most desirable option because of their high specificity and broad selection of targets. However, antibody production, conjugation, and subsequent nanoparticle purification can be extremely costly and time consuming. Immunotherapies such as trastuzumab can cost ~\$70,000 for a full course of treatment (73). Trastuzumab-labeled nanoparticles can easily cost many times this amount because of low nanoparticle conjugation efficiencies alone, which can be <10% (74). Then there is the cost of nanoparticle production, purification, and sterilization to consider. These limitations highlight the need for the continual development of more efficient nanoparticle-antibody coupling strategies. The possibility of lower administered doses and a higher therapeutic index that manifests from the addition of targeting capabilities can also help to offset these costs. Ultimately, however, the motivation to invest in antibody-targeted nanoparticles will likely be driven by the clinical benefits observed with first-generation targeted nanoparticles.

The costs and regulatory hurdles associated with adding imaging capabilities to nanoparticles is likely to be significantly lower than that of targeting. Although theranostic agents can provide insight into tumor accessibility and heterogeneity, this is unlikely to be consistently predictive of tumor response because of a tumor's ability to acquire drug resistance. Therefore, imaging agents that are introduced onto therapeutic nanoparticles are likely to play a supporting role to dedicated imaging agents. In cases where the nanomaterial itself possesses both imaging and therapeutic capabilities, the benefits are likely to be more profound. For example, the ability to identify the distribution of gold nanoparticles within a tumor via CT can help demarcate tumor boundaries and identify poorly perfused necrotic or hypoxic regions. This insight can guide radiation treatment planning and highlights the potential impact that some theranostic agents can have on patient management.

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REVIEW

Soft Matter Models of Developing Tissues and Tumors

David Gonzalez-Rodriguez,^{1*} Karine Guevorkian,^{2*} Stéphane Duezan,^{3*} Françoise Brochard-Wyart^{3†}

Analogies with inert soft condensed matter—such as viscoelastic liquids, pastes, foams, emulsions, colloids, and polymers—can be used to investigate the mechanical response of soft biological tissues to forces. A variety of experimental techniques and biophysical models have exploited these analogies allowing the quantitative characterization of the mechanical properties of model tissues, such as surface tension, elasticity, and viscosity. The framework of soft matter has been successful in explaining a number of dynamical tissue behaviors observed in physiology and development, such as cell sorting, tissue spreading, or the escape of individual cells from a tumor. However, living tissues also exhibit active responses, such as rigidity sensing or cell pulsation, that are absent in inert soft materials. The soft matter models reviewed here have provided valuable insight in understanding morphogenesis and cancer invasion and have set bases for using tissue engineering within medicine.

Soft tissues are complex deformable materials whose rheology is determined by dynamical fluctuations caused by cell activity. These characteristics of biological tissues make

them akin to soft matter, a similarity that has been exploited to investigate tissue mechanics—that is, the movements and reshaping of tissues under the action of forces. The most fruitful anal-

ogy is that between tissues and liquids (*1*) with a viscoelastic rheology (*2*), but specific aspects of tissue behavior have also been explained by analogies with other soft materials, such as viscoelastic pastes (*3*), foams (*4*), emulsions (*5*), colloids (*6*), and polymers.

This article reviews recent work on the application of physical and soft matter concepts to understand how soft tissues respond to forces. We focus our discussion on soft tissues, such as early embryonic tissues or tumors, in which the expression of extracellular matrix molecules that rigidify the tissue is low. Such tissues are well modeled by cellular aggregates, a model system

¹Laboratoire d'Hydrodynamique (LadHyX), CNRS UMR 7646, Ecole Polytechnique, 91128 Palaiseau, France. ²Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS UMR 7104 and INSERM U964, Université de Strasbourg, 67400 Illkirch, France. ³Physico-Chimie Curie, Institut Curie, Université Pierre et Marie Curie and CNRS UMR 168, 26 rue d'Ulm, 75005 Paris, France.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: brochard@curie.fr

reviewed by Lin and Chang (7). We start by discussing the work of Malcolm Steinberg, who made the founding contribution to this research field by proposing the analogy between embryonic tissues and liquids (1, 8). Similarities between tissues and liquids or other soft materials have proven fruitful to quantitatively characterize the rheology of tissues and to explain a number of dynamical tissue behaviors reminiscent of those observed in physiology and development, such as cell sorting or tissue spreading. In spite of the power of such analogies, living tissues additionally display active responses that are not observed in passive soft materials, such as rigidity sensing or cell pulsation. We end the Review by discussing how these advances in tissue mechanics are being applied to understand embryonic morphogenesis, identify determining factors in cancer propagation, and develop novel medical approaches that use tissue engineering.

Steinberg's Differential Adhesion Hypothesis

A liquid is formed by molecules that remain cohesive because of the existence of attractive forces at the molecular scale. At the macroscopic scale, a liquid behaves as a continuous medium, and molecular interactions manifest themselves as the liquid's mechanical properties, such as viscosity (the resistance of the liquid to deformation) and surface tension (the tendency of a liquid to minimize its total surface because of the unfavorable attractive force imbalance experienced by molecules at the liquid's surface). Similar to liquids consisting of molecules with attractive interactions, tissues consist of cells with adhesive interactions (mediated by cell adhesion molecules such as cadherins). Analogous to liquids, the individual size of a cell is much smaller than the typical size of a tissue. Thus, tissues can also be treated as continuous media and their rheology described by macroscopic properties such as surface tension and viscosity, which for tissues crucially depend on intercellular adhesion, among other parameters.

The idea of describing tissues as liquids was inspired by cell sorting experiments. While studying amphibian embryonic development, Hofmeister observed an affinity of alike cells to sort out and associate themselves, which led him to introduce the concept of "tissue affinities" (9). In a seminal 1963 article, Steinberg proposed the Differential Adhesion Hypothesis (DAH) to explain the universal cell sorting behavior through an analogy with the behavior of liquids (1). If oil and water are mixed in a liquid droplet, the two liquid phases separate in order to minimize the total interfacial energy. Similarly, when two different types of cells are randomly mixed forming an aggregate, the two cell populations spontaneously sort out. The final equilibrium configuration minimizes the total surface energy and depends on the surface tensions of the cell populations and their interfacial tension (10). For an interfacial tension smaller than the difference of surface tensions of the two

cell populations, one tissue engulfs the other, with cells having the larger surface tension occupying the interior of the mixed aggregate, whereas for larger interfacial tension, the engulfment is partial. Thus, cell sorting is driven by differences in tissue surface tensions, which are largely determined by differences in expression of cell adhesion molecules, as has been experimentally demonstrated (11, 12). In an experiment on the role of cell adhesion in tissue surface tension, Foty and Steinberg (12) transfected otherwise identical mouse fibroblast cell lines to express different levels of N-, P-, or E-cadherins. The surface tension of aggregates of these cell lines was measured by parallel-plate compression. Independently of the cadherin type, tissue surface tension was found to be linearly proportional to the cadherin expression level. Moreover, cell sorting experiments using heterotypic aggregates of all combinations of the different cell lines systematically produced the cell sorting outcomes predicted by comparing the surface tensions of the different cell populations.

Steinberg's hypothesis that cell sorting in tissues arises from differences in surface tension between different cell populations has gathered extensive experimental support (13) and is now widely accepted. Although energy-minimizing surface tension appears to determine the equilibrium configuration of a tissue, cellular motility and the renewal of continuously bonding and dissociating adhesive junctions are required for a tissue to overcome local energy barriers and progress toward equilibrium. The DAH's postulate that tissue surface tension is determined by the level of expression of cell adhesion molecules remains disputed, and alternative hypotheses have been formulated. The Differential Surface Contraction Hypothesis (DSCH) (14) proposes that surface tension arises from differences in actomyosin-driven cell cortical contractility. In the original formulation of the DSCH proposed by Harris (14), cells with large contractility would strongly tend to minimize their exposed surface on a cell aggregate, thus exhibiting a large tissue surface tension. Exposure of the more contractile cells to the external medium would be very energetically unfavorable, with cell contractility being reduced on heterotypic cell interfaces and further reduced in homotypic cell contacts, thus explaining the spontaneous sorting of cell populations with different contractilities. Experiments of cell sorting in aggregates from different layers of the zebrafish gastrula have yielded partial support for the role of contractility in tissue surface tension. Schötz *et al.* determined that differential adhesion, which is dependent on E-cadherin expression, influences positioning between zebrafish germ layer tissues (15). Krieg *et al.* concluded that tissue surface tension correlated with cell cortical tension [as measured by atomic force microscopy (AFM)] but not with cadherin expression (16). A limitation of this conclusion arises from the use of AFM to measure cortical tension.

AFM measures single-cell responses on time scales of the order of seconds, much smaller than the time scales relevant for tissue surface tension (typically hours), thus making a direct comparison between AFM-measured cortical tension and surface tension unclear. The Differential Interfacial Tension Hypothesis (DITH) (17) combines the DAH and the DSCH to propose that tissue self-arrangements are governed by differences in interfacial tensions, which depend both on cell adhesion and cell contractility. Consistent with this idea, Krieg *et al.* (16) and Manning *et al.* (18) postulated that tissue surface tension is in general determined by both intercellular adhesion and cell cortical tension. Intercellular adhesion increases tissue surface tension, whereas cortical tension does not necessarily increase tissue surface tension (as originally proposed by Harris), but rather it has competing effects on surface tension. This is so because cortical tension not only increases cell-medium tension (which increases tissue surface tension) but it also increases cell-cell tension (which reduces tissue surface tension). The roles of intercellular adhesion and cortical tension in regulating intercellular contacts remain a subject of current research. Maître *et al.* quantified cortex tension at the cell-cell interface by separating cell doublets using dual micropipette aspiration (19). Their results suggest that cortical tension dominates over adhesion in controlling interfacial tension and cell-cell contact expansion, whereas adhesion is needed to mechanically couple the cortices of adhering cells.

Characterization of the Mechanical Properties of Tissue

In order for the DAH to become quantitative, it was necessary to measure the mechanical parameters of tissues. Tissue surface tension is a key parameter to characterize long-term equilibrium conditions, and rheological parameters are required to characterize the dynamic evolution of a tissue under the action of forces. The choice of the appropriate rheological parameters depends on the physical model adopted to describe tissue behavior. Broad experimental evidence indicates that soft tissues behave as elastic solids at short time scales (seconds to minutes) because their short-term deformation is proportional to the applied load and the tissue quickly recovers its initial shape if the load is released. At longer time scales (minutes to hours), tissues undergo cellular reorganizations, which lead to more persistent deformations, usually described experimentally as a viscoelastic behavior (2, 20, 21). The existing experimental observations have led to two paradigms to characterize tissue rheology. The first paradigm postulates that tissues behave as viscoelastic fluids with a surface tension (2, 20, 21), and thus a model tissue is characterized by its surface tension (γ), Young's modulus of elasticity (E), and dynamic viscosity (η) to capture that over long time scales, tissues flow as viscous

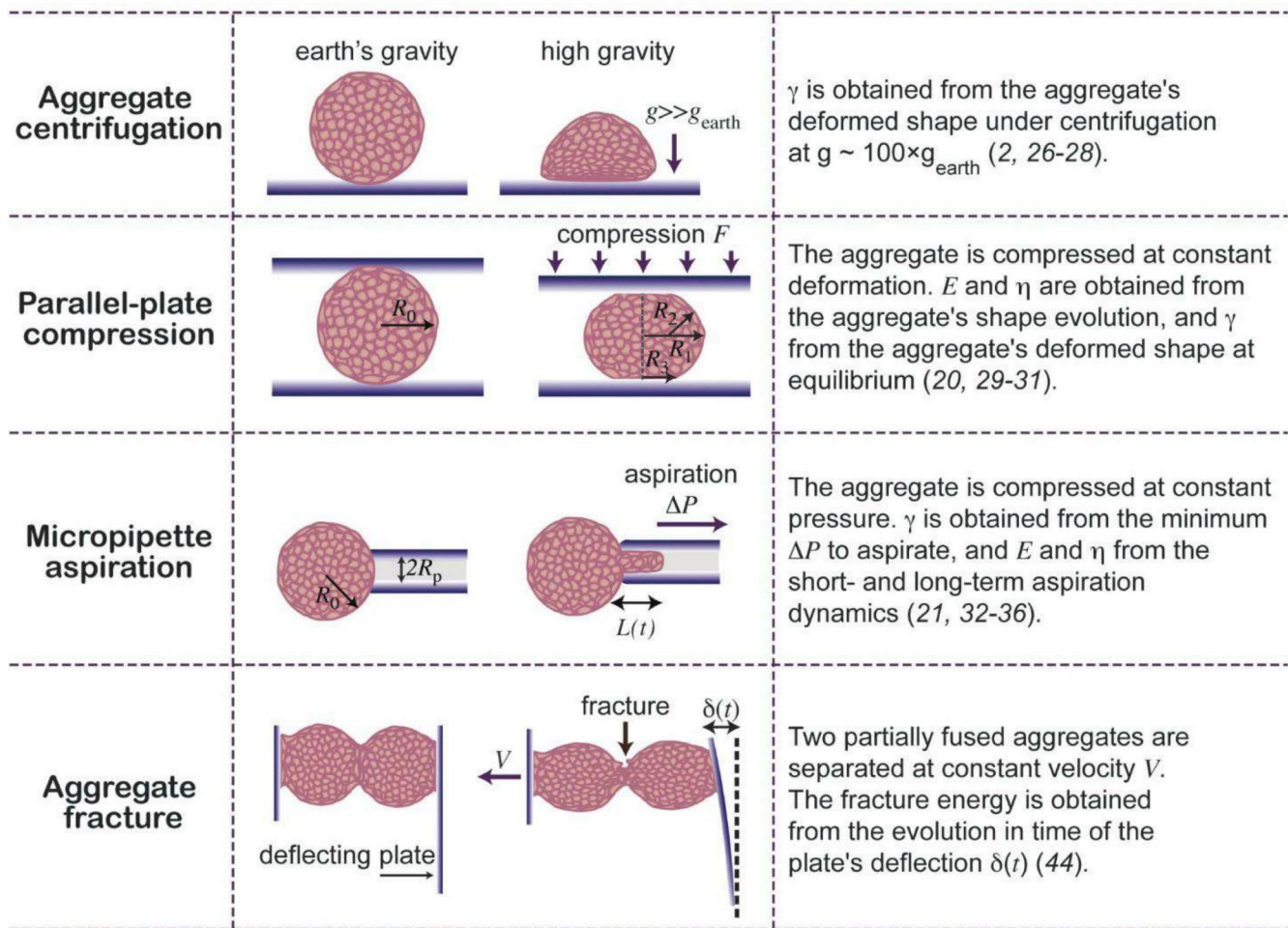


Fig. 1. Primary experimental techniques used to quantify mechanical properties of model tissues.

fluids. The second paradigm describes a tissue as an elasto-visco-plastic solid, characterized by a Young's modulus, a dynamic viscosity, and a yield stress (22–24). The yield stress is a threshold of stress below which the tissue can elastically recover its initial shape after the load is released. According to this second paradigm, if the applied stress is larger than the yield stress the tissue acquires permanent plastic deformations that remain present even after the load is released. In the following, we discuss the main techniques that have been developed to measure tissue mechanical parameters, which are illustrated in Fig. 1; some of these techniques have also been reviewed by Krens and Heisenberg (25).

Phillips and Steinberg proposed characterizing tissue surface tension by using aggregate centrifugation. It consists of subjecting the tissue to a centrifugal field that is a few hundred times stronger than gravity in order to produce measurable deformations (26). When an aggregate is centrifuged for a sufficiently long time, it adopts a flattened shape, which is independent of the initial aggregate shape. The degree of flattening is inversely correlated with the tissue surface ten-

sion. Later improvements of the technique have produced quantitative measurements of the tissue surface tension by using an analysis algorithm of the deformed aggregate shape (27, 28). By following the evolution of the aggregate shape in the centrifugal field (2) or its shape relaxation after centrifugation is stopped, this technique could in principle be used to evaluate the tissue's elastic modulus and viscosity.

To date, the most widely used technique to characterize tissue properties has been parallel-plate compression, introduced by Steinberg and co-workers (20, 29, 30). In this method, an aggregate is placed between two nonadhering parallel plates and compressed to a fixed deformation. A scale measures the evolution of the compression force as a function of time, which allows the determination of the aggregate's viscoelastic properties (η , E). The aggregate eventually reaches an equilibrium shape, analogous to the shape of a liquid drop compressed between two plates. From knowledge of the equilibrium shape of the aggregate and of the corresponding applied force F , the aggregate surface tension can be determined as $\gamma = (1/R_1 + 1/R_2)^{-1}F/(\pi R_3^2)$, where R_1

and R_2 are the principal radii of curvature of the deformed aggregate and πR_3^2 is the contact area between the aggregate and either of the plates (Fig. 1) (29). The value of the surface tension is very sensitive to an accurate determination of the aggregate's equilibrium shape, which can be experimentally challenging (31).

Micropipette aspiration is a more recently introduced technique to characterize tissue rheology (21, 32–36). In the method proposed by Guevorkian *et al.* (21), an aggregate is aspirated at constant suction pressure into a micropipette of smaller diameter than that of the aggregate, and the length of the aspirated tongue, $L(t)$, is tracked with time. For the aggregate to be aspirated, the applied suction pressure must be larger than a critical aspiration pressure ΔP_c , related to the aggregate's surface tension by $\gamma = (1/R_p - 1/R_0)^{-1}\Delta P_c/2$, where R_p and R_0 are the micropipette and aggregate radii, respectively. After aspirating the aggregate for a long enough time to capture its long-term behavior, the pressure is released, causing the aggregate to retract out of the pipette under the action of its surface tension. By fitting the aspiration and retraction curves

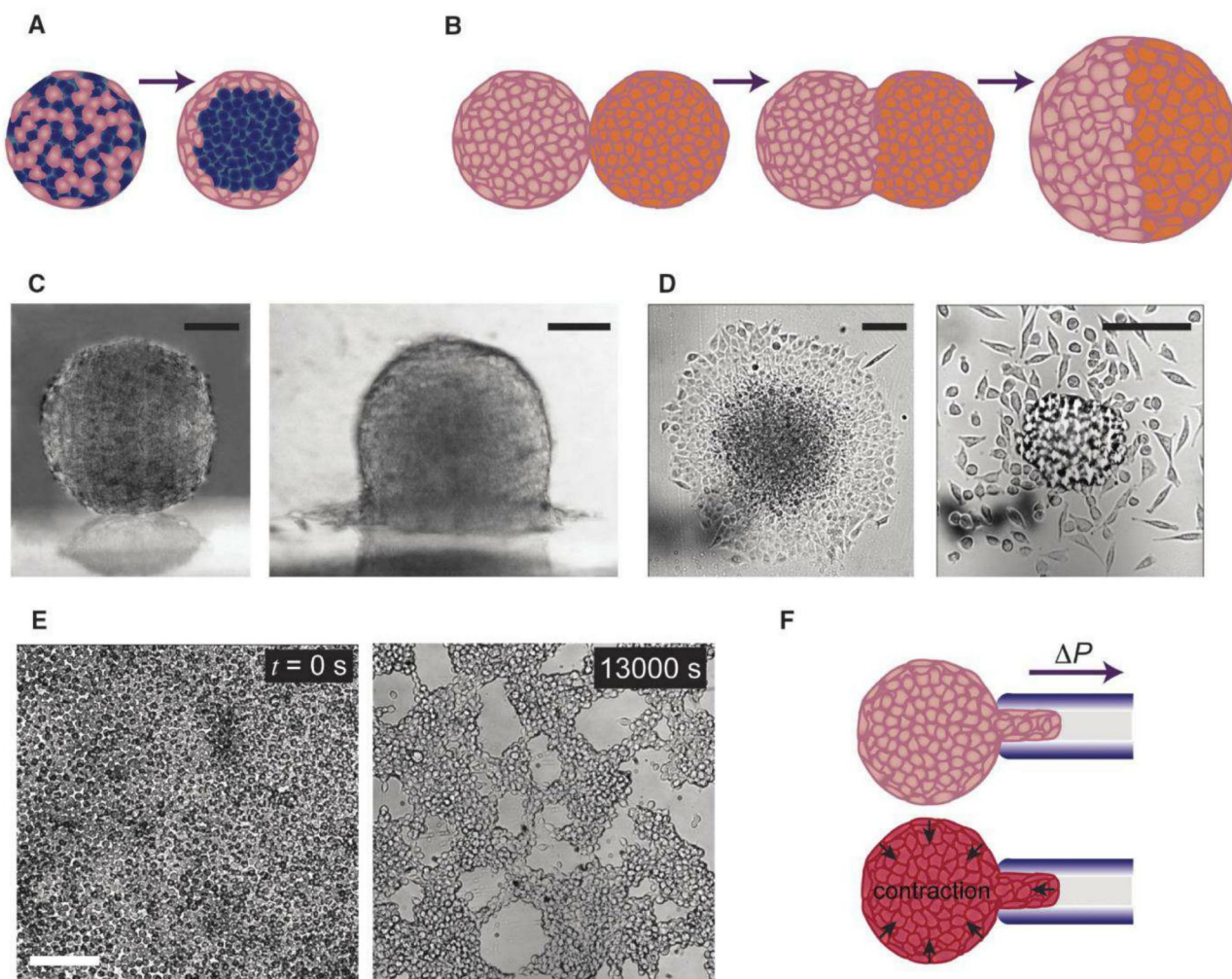


Fig. 2. Wetting analogies of tissue behavior. **(A)** Cell sorting. Two different cell populations (pink and blue) in a cell aggregate spontaneously sort out according to their surface and interfacial tensions. **(B)** Aggregate fusion. When brought into contact, two aggregates of the same cell line fuse to yield a larger, spherical aggregate. **(C)** Aggregate spreading on a wettable substrate. Reprinted with permission from (57). **(D)** Long-term spreading dynamics. As the aggregate cohesivity is de-

creased, the dynamics of the precursor film transitions from a liquid (left) to a 2D gas (right). Reprinted with permission from (3). **(E)** Dewetting of a cellular monolayer on a nonwettable substrate, a phenomenon opposite to spreading. Reprinted with kind permission of The European Physical Journal (EPJ) from (6). Scale bars, 100 μm . **(F)** Aggregate shivering. Active pulsatile contractions are observed during micropipette aspiration when the aspiration pressure is within a certain range.

to a viscoelastic model, the aggregate's elastic modulus, viscosity, and surface tension are determined. This technique has the advantage of a relatively simple experimental setup and image analysis. Using this technique on aggregates of mouse sarcoma cell lines expressing E-cadherin, Guevorkian *et al.* reported that the aggregate's surface tension increased with the applied pressure (21), which could indicate an active reinforcement of the aggregate in response to the applied stress. Such a reinforcement has not been reported in parallel-plate compression experiments. Although in parallel-plate compression aggregates are compressed to a constant strain, with the exerted stresses being relaxed over a typical time scale of about an hour, in micropipette aspiration aggregates undergo a continuous traction at constant stress. It has been reported that traction forces elicit active cell contractile responses (37, 38), which

may not arise during compression. A systematic study comparing parallel-plate compression and micropipette aspiration on aggregates of the same cell lines would help to clarify the effect of these differences. It has also been pointed out that whereas in parallel-plate compression the whole aggregate is stressed, micropipette aspiration probes only a subset of the aggregate's cells (39). The number of cells subjected to stress scales as R_p^3 . Thus, for the continuum hypothesis to be valid and the technique applicable to characterize aggregate rheology, the micropipette radius should be large enough to probe a few hundreds of cells, which is usually accomplished with $R_p \approx 30 \mu\text{m}$.

The existence of a yield modulus above which aggregates exhibit plastic behavior remains a matter of controversy. Some authors have postulated the existence of a yield modulus in tissues, which physically would arise from the critical force

required to break intercellular bonds and induce cellular reorganization (22). To our knowledge, no systematic experimental investigation of the existence of a yield stress in tissues has been conducted. Existing experimental observations such as the spontaneous fusion of two aggregates (40) seem to suggest that the yield stress, if it exists, is smaller than the stresses induced by tissue surface tension, and thus deformations are relaxed by surface tension over a time scale of the order of $\eta R/\gamma$, where R is the characteristic aggregate size (typically a few hundred micrometers). Moreover, bond formation and dissociation is more accurately described as a dynamic process, where the formation and dissociation rates vary continuously with the applied load. An appealing solution to the debate about the existence of tissue plasticity has been suggested by Marmottant *et al.* (41), who proposed that the

rheological description of tissue behavior depends on the time scale considered. When the applied stress is larger than the typical energy barrier for cell rearrangement, stresses are relaxed fast. When the residual stresses become small compared with energy barriers, they are relaxed by fluctuations only, and thus stress relaxation substantially slows down. In short-time observations, residual stresses can be described as tissue plasticity, even if they are eventually relaxed at long enough times. Depending on the cell line, the time for complete stress relaxation may be minutes, hours, or even days. Accurate measurement of tissue surface tension by parallel-plate compression, which assumes that equilibrium conditions have been attained, requires that the time allowed for tissue relaxation is long enough to completely dissipate residual stresses (41). A definitive answer to the debate of characterizing tissues as either viscoelastic fluids or visco-elasto-plastic solids could come from measuring the frequency response of tissues to a periodic forcing, a much-needed experiment that to our knowledge has not previously been reported. Different constitutive models (such as the viscoelastic Maxwell model, the generalized viscoelastic Kelvin model, or the elasto-plastic Bingham fluid) predict different frequency responses. Thus, periodic forcing experiments could be used to determine the appropriate constitutive model for soft tissues and whether the same constitutive model with different parameter values can describe different types of tissue, or rather whether different models are needed for different tissues.

At time scales comparable with the time for cell division (usually of the order of a day), cell proliferation and apoptosis affect aggregate rheology. A theoretical model by Ranft *et al.* (42) suggests that cell rearrangements induced by cell division and apoptosis will result in a viscous tissue response, independent of the adhesion-mediated viscosity. At long time scales, both components of tissue viscosity must be accounted for. Moreover, unlike adhesion-mediated viscosity, proliferation-controlled viscosity is affected by the isotropic pressure applied on the cells because high pressures have experimentally been shown to inhibit cell proliferation (43).

Last, another type of experimental technique characterizes the resistance of a model tissue to stress-induced failure, either by internal fracture (44) or by detachment from a substrate modeling the extracellular matrix. By pulling apart two

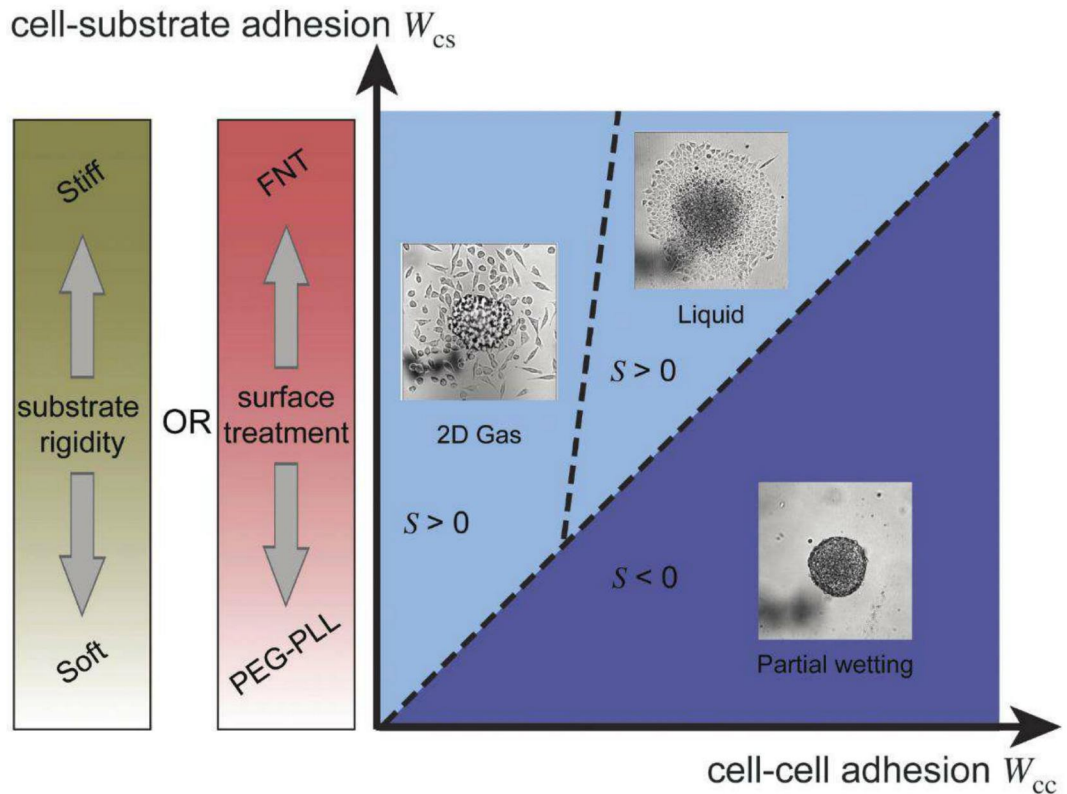


Fig. 3. Phase diagram of tissue spreading. The aggregate's fate is governed by a competition between the cell-substrate adhesion energy W_{cs} and the cell-cell adhesion energy W_{cc} , the difference of which sets the sign of the spreading parameter $S = W_{cs} - W_{cc}$. The images show the long-term fate of the aggregate in each of the regions. The cell-substrate energy W_{cs} can be controlled either by modifying the substrate's surface chemistry [nonadhesive PEG-poly-L-lysine (PEG-PLL) versus adhesive fibronectin (FNT)] or its rigidity (soft versus stiff). The two top images are reprinted with permission from (3).

partially fused aggregates at constant speed and measuring the fracture energy, recent experiments in our laboratory suggest an analogy between tissues and polymers. Similar to polymers, the resistance of tissues to fracture appears to depend on the imposed deformation rate.

Wetting Analogies of Tissue Behavior

Analogies between tissue mechanics and dynamical phenomena involving liquid interfaces, known as wetting phenomena, have been used to explain several ubiquitous tissue behaviors. Among such behaviors, cell sorting (illustrated in Fig. 2A) has received substantial attention because it is a model phenomenon for the mutual envelopment of different tissue layers during morphogenesis (1). Graner and Glazier (45, 46) developed a two-dimensional (2D) cellular Potts model to investigate the physics of cell sorting. By accounting for differential adhesion and for random cell fluctuations associated with cell activity, their model was able to reproduce cell sorting. The model predicts the existence of two regimes in the evolution of an aggregate formed by two randomly mixed cell populations. The fast, short-time regime leads to a partial cell sorting

state, whereas the slower, long-time regime leads to complete cell sorting. Both the partial and complete cell sorting states have been observed experimentally.

The tendency of cellular aggregates to spontaneously round up and acquire a spherical shape, which is an effect of the area-minimizing surface tension, is analogous to the behavior of liquid drops. The dynamics of rounding up have been experimentally studied in 2D hydra aggregates, which spontaneously evolve from an elliptic to a circular shape (47), and in 3D chick embryo aggregates, which evolve from an irregular to a spherical shape (48). Both phenomena are reasonably well described by modeling an aggregate as a viscoelastic liquid drop with a surface tension.

Fusion of two cell aggregates (Fig. 2B) is analogous to the coalescence of two viscous liquid drops. The dynamics of spontaneous aggregate fusion (40, 49) are well described by a balance between the energy gain by reducing the surface area, which is driven by surface tension, and the energy dissipation due to internal viscous friction. Tissue phenomena governed by a balance between surface tension (γ) and viscosity (η), such as tissue envelopment, rounding up,

or fusion, are completed over a characteristic time scale given by $R\eta/\gamma$, where R is the final aggregate radius and γ/η is a typical velocity of the order of 10^{-8} m/s, which yields a typical time scale of several hours.

A striking analogy between tissue mechanics and liquid wetting is found in tissue spreading (Fig. 2C) (3, 50). As with liquid wetting, the aggregate's fate is determined by the sign of the spreading parameter $S = W_{cs} - W_{cc}$, where W_{cs} and W_{cc} are the cell-substrate and cell-cell adhesion energies per unit area, respectively. These adhesion energies are closely related to surface and interfacial tensions ($W_{cc} = 2\gamma$ and $W_{cs} = \gamma_{sm} + \gamma - \gamma_{cs}$, with γ_{cs} , γ_{sm} , and $\gamma = \gamma_{cm}$ being the cell-substrate, substrate-medium, and cell-medium interfacial tensions, respectively). The analogy between tissue spreading and liquid wetting is most remarkable because cells use distinct machineries to adhere to one another (typically involving cadherin pathways) and to adhere to substrates (typically involving integrins). This is in contrast to inert soft matter wetting systems, in which surface energies are all driven by the same physics of molecular interactions. Although this wetting analogy is supported by experimental evidence, there could be some tissue types with strong feedback between cadherin and integrin pathways in which the analogy breaks down or the wetting behavior changes as a function of time. The effect of varying cell-cell and cell-substrate adhesion energy on aggregate spreading is illustrated in Fig. 3. If $S < 0$, the cell-cell adhesion energy is larger than the cell-substrate adhesion energy. This is a nonwettable surface on which the aggregate remains spherical, a state termed "partial wetting" because the aggregate exhibits a finite contact angle with the surface. Partial wetting is experimentally observed when a cell aggregate is placed on a polyethylene glycol (PEG)-coated substrate. In contrast, if $S > 0$, cell-substrate contact is energetically favorable, and the aggregate spreads on the surface, such as when a cell aggregate is placed on a fibronectin-coated substrate. The dynamical laws governing aggregate spreading are the same as for a viscoelastic paste (3). At long times (Fig. 2D), a striking new analogy to liquid wetting is observed (3): A precursor film of cells spreads around the aggregate. In strongly cohesive aggregates (large W_{cc}), the film is a continuous cellular monolayer, whereas in weakly cohesive aggregates individual cells can escape from the aggregate. This is reminiscent of a wetting transition from a liquid to a 2D gas. By using aggregates of mouse sarcoma cell lines expressing different E-cadherin levels, Douezan *et al.* (3) demonstrated that this liquid-to-gas transition in aggregates is induced by reducing cadherin expression. This suggests that the epithelial-mesenchymal transition, which is associated with a reduction of cadherin expression and plays important roles in embryonic development and tumor metastasis, may be interpreted as a wetting transition. More-

over, knowledge of the dynamical laws governing tissue spreading allows to quantitatively characterize the action of drugs in inhibiting the spreading of tumors (51).

When a liquid film is placed on a nonwettable substrate, it retracts from the substrate by breaking down into isolated drops, such as occurs to the water film on one's skin when coming out of the shower. This phenomenon, called dewetting, has also been observed in cellular systems (6, 52). When a confluent layer of cells is placed on a PEG-coated substrate, the cells spontaneously withdraw from the surface to form isolated aggregates, following the same dynamical law as viscous liquid dewetting (Fig. 2E) (6). If initially the cells are subconfluent, they migrate on the surface following collectively a diffusive law. The cells eventually meet and form 3D aggregates, according to the same dynamical law as the diffusion-limited aggregation of colloids (49).

Mechanotransduction in Tissue Mechanics

Because tissues are composed of living cells capable of sensing and reacting to forces, they exhibit active mechanotransduction phenomena that distinguish them from passive materials. An important example of this active behavior is the ability of tissues to sense and react to substrate rigidity. As the substrate becomes stiffer, cells show increased contractile traction forces, more stable focal adhesions, better defined actin stress fibers, and increased adhesion strength (53–56). In terms of the wetting analogy of tissue spreading discussed above, as the substrate becomes stiffer, cell-substrate adhesion becomes more favorable, and W_{cs} increases (Fig. 3). It has been experimentally shown that the rigidity of the substrate can induce a wetting transition from partial wetting ($S < 0$) below a threshold of substrate rigidity (E_c) to complete wetting ($S > 0$) above this rigidity threshold (57). Unlike the statics of passive liquid wetting, which are only affected by substrate rigidity if the substrate is deformable enough to form a wetting ridge around the liquid drop (58), the rigidity-induced wetting transition in cellular aggregates is observed without appreciable deformation of the substrate. The dynamics of spreading and cell diffusion from the aggregate are also affected by substrate rigidity. On a substrate rigid enough to induce complete wetting, the long-term spreading dynamics follows a diffusive law with a diffusion coefficient that depends on the rigidity, with a maximum for $E \approx 2E_c$ (57). If the experiment starts from a cell layer spread on a soft substrate, cells dewet the substrate and form 3D aggregates, whereas on a rigid substrate, cells do not aggregate (6, 59). This rigidity-dependent wetting suggests a physical explanation of why cancer invasion is stimulated by increased connective tissue stiffness, an effect known as desmoplasia and observed, for example, in breast cancer and pancreatic adenocarcinoma (59).

Tissue pulsation is another active phenomenon that plays important roles in embryonic development (60). When a cellular aggregate is aspirated into a micropipette within a precise range of aspiration pressures, pulsatile tissue contractions are observed, a phenomenon that has been termed "aggregate shivering" (Fig. 2F) (38). Aggregate shivering has also been observed when an aggregate is stretched between two plates but not in parallel-plate compression. Shivering has been explained as an active contraction of the cells in the aggregate after they have been stretched beyond a certain threshold. The existence of a time delay between stretching and active cell contractile response quantitatively explains the characteristic time between shivering contractions (38) as well as the pulsation of amnioserosa cells observed during dorsal closure in the embryonic development of *Drosophila* (61).

Another active response of tissues to forces is the hyperrestoration principle proposed by Belousov *et al.* (62). This principle conjectures that when a cell or tissue is shifted from its mechanical equilibrium by an external force, it develops an active response directed toward restoration of the initial condition. However, the restorative response generally overshoots the mere compensation of the effect caused by the external force (hyperrestoration). Belousov and Luchinskaia experimentally illustrated this principle by stretching *Xenopus* tissue explants (63). They reported that, once the stretching force ceases, the tissue, instead of simply relaxing forces, continues elongating and exhibits an extensive shape reorganization.

Applications to Physiology and Bioengineering

The knowledge in tissue mechanics gained through analogies with soft matter has been applied to understand important phenomena in physiology. For example, Winters *et al.* showed an inverse correlation between the surface tension of a tumor and its invasive potential (64). They determined the in vitro surface tension of malignant astrocytoma cell lines by using the parallel-plate compression technique and their cell invasiveness by using a matrigel transfilter invasion assay. They found that the relevant parameter to determine tumor invasiveness is the surface tension and not simply cadherin expression. In their experiments, surface tension did not correlate with the N-cadherin expression level, which they attribute to an effect on surface tension of cell interaction with the extracellular matrix. The effect of extracellular matrix interactions on tumor progression was further investigated by Hegedus *et al.* (65), who showed that tumor-invasive patterns depend on a balance between cell-cell interactions (mediated by cadherins) and cell-matrix interactions (mediated by integrins as well as by metalloprotease activity), and also by Sabari *et al.* (51), who showed that assembly of a fibronectin

extracellular matrix slows down or suppresses spreading of an aggregate of brain tumor cells.

Tissue wetting analogies have broad implications to embryonic development. Cell sorting plays a role in zebrafish and *Xenopus* gastrulation, mouse blastocyst formation, or chick limb bud formation, as reviewed by Krens and Heisenberg (25). Although the direct applicability of in vitro cell sorting behaviors to explain embryonic tissue layering remains debated (66), sorting patterns of embryonic tissues observed in vivo can often be reproduced in vitro by using cellular aggregates, as demonstrated, for example, with the organization of brain cortical layers in mice (67). Aggregate fusion is found, for example, in early chick heart development, in which septa and valves form by fusion of endocardic swellings known as cardiac cushions (40). Viscous tissue spreading is a phenomenon ubiquitously found in morphogenesis whenever tissue layers rearrange their relative positions, such as during embryonic epiboly (68). The power of analogies to liquid systems is not restricted to 3D geometries. In the 2D cell patterning observed in the retinal epithelium of *Drosophila* (4, 69), cone cells arrange themselves following the same energy-minimization principles that govern the organization of soap bubbles in a 2D liquid film. Similarly, the evolution of the configurational pattern of cells forming the wing disc epithelial layer in *Drosophila* can be explained by global energy minimization of a system governed by the interaction between line tension, cell elasticity, and cell contractility (70).

Understanding tissue mechanics provides the basis to tissue engineering, which aims to develop new strategies of medical treatment based on artificial tissue regeneration. In building artificial tissues, surface tension can be used to induce the spontaneous assembly and organization of cells in analogous configurations as those found in vivo. This is exemplified by the spontaneous in vitro self-assembly of pancreatic islet cells, which are capable of reproducing a realistic islet topology (71). Using surface tension to induce self-organization is one of the bases of the bioprinting techniques developed by Forgacs and co-workers (72). In contrast with traditional tissue engineering methods, which propose the implantation of a tissue scaffold seeded with cells, bioprinting is a scaffold-free approach that uses the liquid-like fusion and wetting properties of cellular aggregates to form organized 3D tissue structures. Closely placed aggregates fuse and form larger structures, whereas undesired aggregate spreading is prevented by the use of nonwetable substrates (73, 74). Forgacs and co-workers have developed two different bioprinting approaches to produce 3D structures. In the first approach, a modified inkjet printer uses cellular aggregates as “ink drops” and deposits them layer by layer with collagen sheets as support. Then, spontaneous fusion of the cell aggregate ink drops produces 3D tissue structures (74). The second approach, devised for the

assembly of long structures such as blood vessels, uses the inkjet printer to assemble the aggregates into 1D cylinders, corresponding, for example, to one longitudinal section of the vessel wall. These cell cylinders are appropriately placed in 3D space with the help of cylinders of agarose, a nonwetable biocompatible material, which eventually are removed to produce void spaces such as the vessel lumen (75). An alternative technique to assemble blood vessels has been proposed by Fleming *et al.* (76). This technique uses uniluminal spheroids, which are shell-like aggregate structures formed by an outer layer of smooth muscle cells, an inner layer of endothelial cells, and an internal space void of cells. Fusion of uniluminal spheroids yields elongated vessel-like tubes, a process that can be applied to engineer blood vessels.

In conclusion, soft matter physics has been fruitfully applied to quantitatively explain fundamental phenomena in tissue mechanics, such as cell sorting or tissue spreading. These advances in our fundamental knowledge of tissue mechanics are being applied to understand embryonic morphogenesis and cancer progression, and they are playing a crucial role in the development of the new field of tissue engineering. Several key questions in the field remain open. The rheology of soft tissues has not been conclusively described; in particular, the concept of tissue plasticity remains debated. Future experiments that allow discrimination between different constitutive models should be designed, such as investigations of the frequency response of tissues to forces. More generally, models of tissue mechanics have often focused on partial descriptions of tissue behavior that are successful in explaining specific features of a type of tissue at a certain scale. Future modeling efforts should discuss the general applicability of theoretical models to different tissues, as well as link the physics at different scales to provide a comprehensive view of tissue mechanics. Specifically, future studies should address how the macroscopically measurable tissue viscosity arises from and depends on biomolecular mechanisms, an important question that remains largely unexplored. A promising area of further applicability of soft matter concepts is the study of instabilities in which tissue surface tension plays a role, such as in epithelial dysplasia or cancerous invasion (77). Last, new and rapidly developing 3D cell culture techniques provide novel in vitro systems in which to study tissue mechanics. The closer proximity of these systems to in vivo conditions will help bridge existing gaps between observed in vitro behaviors and physiological phenomena.

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REVIEW

Unlike Bone, Cartilage Regeneration Remains Elusive

Daniel J. Huey, Jerry C. Hu, Kyriacos A. Athanasiou*

Articular cartilage was predicted to be one of the first tissues to successfully be regenerated, but this proved incorrect. In contrast, bone (but also vasculature and cardiac tissues) has seen numerous successful reparative approaches, despite consisting of multiple cell and tissue types and, thus, possessing more complex design requirements. Here, we use bone-regeneration successes to highlight cartilage-regeneration challenges: such as selecting appropriate cell sources and scaffolds, creating biomechanically suitable tissues, and integrating to native tissue. We also discuss technologies that can address the hurdles of engineering a tissue possessing mechanical properties that are unmatched in human-made materials and functioning in environments unfavorable to neotissue growth.

Nearly two decades ago, the concept of tissue engineering promised healing of damaged tissues and organs via the use of living, functional constructs. By manipulating cells, scaffolds, and stimuli, the premise was that tissues could be generated that, upon implantation, would integrate to native tissues and restore functions lost due to trauma, disease, or aging (1). Tissue engineers recognized that the first targets would be tissues with homogeneous structure and few cell types (2). Due to diffusion limitations, it was also anticipated that these would be thin, avascular tissues. Thus, the first cell-based products would be for skin and articular cartilage due to their almost two-dimensional nature. However, despite its more complex composition, including the presence of multiple cell types and vascularity, bone exhibits a high level of innate repair capability that is not present in cartilage. Hence, bone tissue, rather than cartilage, has seen more development as a target for regeneration.

Articular cartilage is the elegantly organized tissue that allows for smooth motion in diarthrodial joints. Our bodies possess a number of dis-

tinct cartilages: the hyaline cartilages of the nasal septum, tracheal rings, and ribs; the elastic cartilages of the ear and epiglottis; and the fibrocartilages of the intervertebral discs, temporomandibular joint disc, and knee meniscus. Articular cartilage is distinct in its weight-bearing and low-friction capabilities. Damage to this tissue can impair joint function, leading to disability. Unlike the majority of tissues, articular cartilage is avascular. Without access to abundant nutrients or circulating progenitor cells and by possessing a nearly acellular nature, cartilage lacks innate abilities to mount a sufficient healing response (Fig. 1). Thus, damaged tissue is not replaced with functional tissue, requiring surgical intervention (3). Traditional techniques for cartilage repair include marrow stimulation, allografts, and autografts (Fig. 2). Although successful in some aspects, each of these techniques has limitations. Marrow stimulation results in fibrocartilage of inferior quality that does not persist; allografts suffer from lack of integration, loss of cell viability due to graft storage, and concerns of disease transmission; and autografts also lack integration and require additional defects (3).

Limitations of Making an Engineered Cartilage for Clinical Use

For load-bearing tissues, correlations between structure and function must be understood to

establish tissue engineering design criteria. Cartilage's viscoelastic properties manifest from its extracellular matrix (ECM) composition of water (70 to 80%), collagen (50 to 75%), and glycosaminoglycans (GAGs) (15 to 30%) (3). This composition provides cartilage with compressive, tensile, and frictional properties that enable survival and function within the biomechanically arduous joint environment.

Successful methods to regenerate bone, but not cartilage, stem from a discrepancy between the innate repair responses of these two types of tissue (Fig. 1) (4). A large number of cells (osteoclasts and osteoblasts) are involved in perpetual bone breakdown and remodeling. Also, the periosteum and bone marrow contain stem cells that can differentiate into bone-producing cells. Bone's extensive vascularity provides abundant nutrients and blood-borne proteins that stimulate tissue repair. Defects in bone can thus be self-repaired up to a critical size, although regeneration in large bony defects requiring vascularization continues to be a problem. In contrast to bone's cellularity, cartilage's few cells exhibit low metabolic activity. Its scarce resident stem cells, which have recently been identified, appear to require considerable in vitro manipulation to produce cartilage (3, 5). Few, if any, cells are specialized in cartilage remodeling; chondrocytes have only been described for calcified or hypertrophic matrices. Cartilage is dependent on synovial fluid perfusion to meet its nutritional needs. Without cells and factors conducive to healing, even small, superficial cartilage defects fail to heal (3).

Current bone-regeneration products are used in cases where external support is provided by plates or cages or where the implant is not intrinsic to the stability of the bony structure. These indications allow sufficient mechanotransduction for stimulation of bone growth and, thus, successful bone regeneration, without necessarily recapitulating native biomechanical properties. For cartilage, comparable indications do not exist, and the generated tissue must be strong, yet highly deformable, and lubricious while exhibiting time-dependency in its stress-strain response. Cartilage's biomechanical environment, consisting of forces over a large range of motion, can take

Department of Biomedical Engineering, University of California, Davis, 1 Shields Avenue, Davis, CA 95616, USA.

*To whom correspondence should be addressed. E-mail: athanasiou@ucdavis.edu

a devastating toll on neocartilage lacking adequate biomechanical properties.

Cell Types for Cartilage Regeneration

Whether it is stem cells or terminally differentiated cells, the most important selection criterion is the ability to produce tissue-specific ECM. Secondary criteria include ease of acquisition and induction toward the desired phenotype. For bone regeneration, mesenchymal stem cells (MSCs) fulfill the desired criteria, so other cells are seldom used (6). For cartilage, neither MSCs nor chondrocytes, the resident cells of cartilage, have shown this same degree of success.

In vivo, periosteum and bone marrow-derived MSCs migrate to repair small defects (Fig. 1) (4). For defects too large to heal naturally (critical size defects), bone repair employing in situ MSCs can be augmented by osteoconductive or osteoinductive scaffolds with or without growth factors. Isolated MSCs, from bone marrow or adipose tissue [termed ASCs (7)], also retain the ability to create bone in vitro (6).

The use of MSCs in cartilage regeneration includes microfracture, cell slurry or construct im-

plantation, and recruitment from the synovial membrane (Fig. 2) (8). MSCs can differentiate into numerous cell types—including chondrocytes, fibrochondrocytes, and hypertrophic chondrocytes—resulting in a mixture of cartilaginous, fibrous, and hypertrophic tissues (9, 10). Despite short-term clinical success, this repair tissue eventually fails, as it does not possess functional mechanical properties. For example, even though fibrocartilaginous repair tissue from microfracture results in initially enhanced clinical knee-function scores, over 2 years it degrades, and scores decline (9). Scaffolds used with microfracture enhance hyaline quality and increase fill percentage, but fibrous tissue still results (11). In conjunction with matrix formation, MSC anti-inflammatory effects may be important in alleviating symptoms (12). If in situ MSC differentiation is insufficient for long-term efficacy, can in vitro manipulation yield hyaline tissues? Unfortunately, chondro-differentiation of MSCs results in an unnatural differentiation pathway that is unlike either endochondral ossification or permanent cartilage formation in that markers of hyaline cartilage (collagen type II and SOX-9), hypertrophy (collagen type X and MMP13), and

bone (osteopontin and bone sialoprotein) are expressed concurrently (13). The success of MSC-based techniques may remain limited if the presence of fibrous and hypertrophic tissue cannot be eliminated.

Therapies employing autologous chondrocytes suffer from requiring two surgeries (for cell harvest and implantation) and forming fibrous repair tissue. It may seem counterintuitive that the constituent cells of cartilage cannot produce a purely hyaline tissue. However, to obtain sufficient chondrocyte numbers for therapy, the required in vitro expansion induces dedifferentiation. Expansion outside of the natural biochemical and biomechanical milieu results in a fibroblastic phenotype, as evidenced in autologous chondrocyte implantation (ACI) procedures (Fig. 2), in which more than 90% of the repair tissue is fibrocartilaginous (14). Research in chondrocyte redifferentiation in vitro has resulted in several products under development that yield hyaline-like neotissue in 27 to 77% of biopsies (15, 16). To further increase the amount of hyaline repair tissue, other products use younger (more chondrogenic) allogeneic chondrocytes, omit the scaffold material, and

apply physiologically inspired stimuli (17, 18).

Allogeneic and xenogeneic cells are also investigated, because cartilage is perceived as immunoprivileged (3). The concept that a dense matrix protects transplanted chondrocytes is bolstered by the fact that fresh osteochondral allografts restore function without antigen matching, extensive processing, or immunosuppressives. The seemingly impenetrable matrix is nonetheless subject to immune-related breakdown, as is evident in inflammatory arthritis. Also, cartilage-repair studies often use nonquantitative assessments (such as swelling) to examine immunogenicity; quantity and type of immune cells, cytokines, and metalloproteinases directed against the implant are seldom presented. This is also true for human osteochondral allografts; few systemic assessments of the immune response exist. More data need to be gathered for both osteochondral allografts and engineered cartilage before one can conclude that nonautologous cells are acceptable.

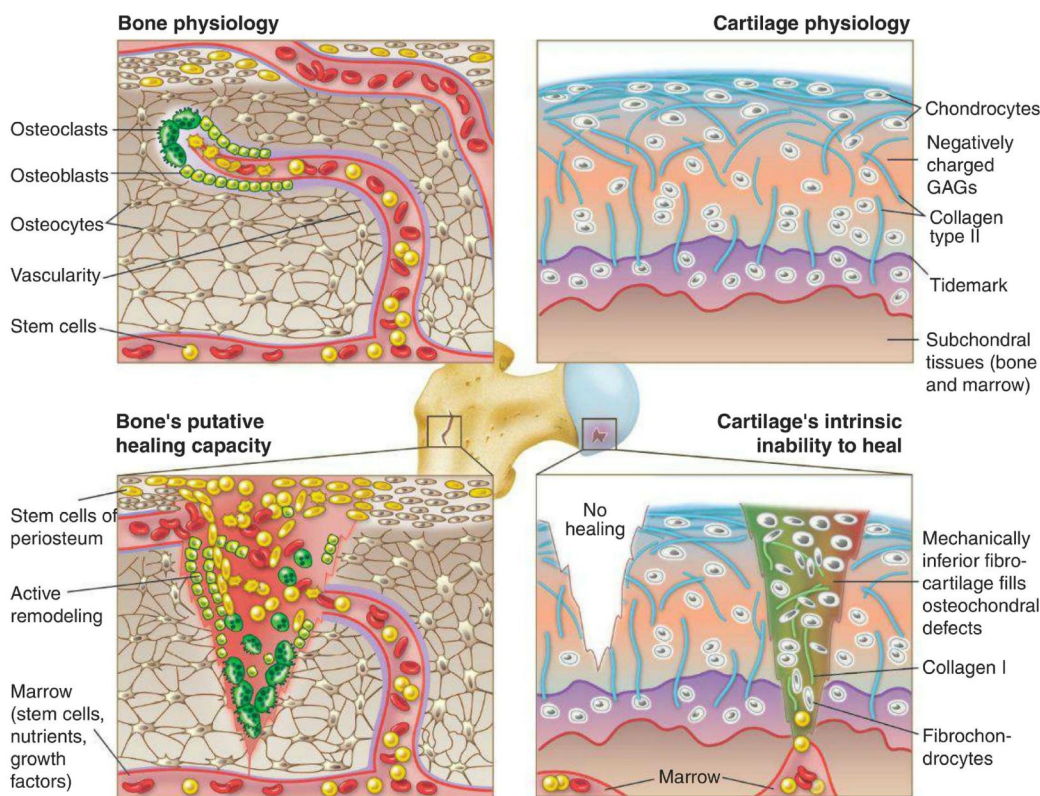


Fig. 1. Differences in the physiologic environment, metabolic rate, and cellular make-up of bone and cartilage have profound effects on the potential to engineer these tissues. The large articulating motion experienced by cartilage, but not bone, can damage newly developed tissues that do not possess required lubrication, compressive, or tensile properties. Additionally, cartilage's hyaline, nonadhesive nature precludes integration, whereas bone integrates rapidly, even with metal. Through the presence of stem cells in marrow and in the periosteum, as well as access to abundant nutrients via vasculature, bone possesses inherent reparative capability that can be harnessed in regenerative therapies. Cartilage's hypocellularity and lack of nutrient supply, coupled with the inability of bone marrow MSCs or resident chondroprogenitor cells to generate hyaline ECM, result in a tissue unable to mount a functional healing response. Thus, in contrast to bone's ability to heal, cartilage needs more robust exogenous approaches to achieve satisfactory regeneration.

Are Scaffolds Required for Cartilage Synthesis?

In utero, tissue development occurs without exogenous scaffolds: Through cell-cell contact, chemical secretion, and, in the case of cartilage, mechanical forces, cells self-organize into differentiated tissues.

In vitro, many tissue engineering strategies have been structured around scaffold design to direct organization and differentiation. For example, collagen sponge, impregnated with hydroxyapatite and bone morphogenetic protein-2, is used clinically with success by facilitating MSC colonization and osteogenic differentiation (19). A general consensus regarding scaffold material for bone has been reached (e.g., combinations of collagen, hydroxyapatite, and tricalcium phosphate), but a large variety of materials are still under assessment for cartilage regeneration (4).

Considerations for scaffold design in cartilage engineering include: (i) matching biodegradation and growth rates (4), (ii) removing degradation by-products (e.g., acidic molecules from polymer degradation can provoke degeneration), (iii) removing harsh chemicals involved with scaffold fabrication, and (iv) designing scaffolds to maintain spherical chondrocyte morphology and phenotype (20). Most scaffolds, perhaps with the exception of hydrogels, promote cell spreading, which encourages fibrous matrix production (20, 21). Also, (v) matching scaffold and native

cartilage compressive properties may be crucial, as stiff scaffolds shield mechanosensitive cartilage-forming cells from experiencing loading, whereas soft scaffolds may fail upon implantation (22). Additionally, (vi) scaffolds must possess sufficient surface and tensile properties for functioning in the high shear joint environment. Insufficiencies in these properties result in wear to opposing or adjacent cartilage due to abrasive contact with the articular surface or third body wear from sheared-off scaffold debris. As these considerations are difficult to overcome, it may be suggested that scaffolds be omitted from cartilage engineering.

To mitigate the challenges associated with scaffold use, techniques promoting formation of biomechanically robust neocartilage without using scaffolds have been developed (3). As these techniques allow the cells to take on a rounded morphology, characteristic of the chondrogenic phenotype, scaffoldless techniques were initially used to form small spherical aggregates for studying chondrogenesis. Recent research has expanded the use of scaffoldless techniques to the generation of cartilage constructs. For exam-

ple, by presenting only nonadherent surfaces to the cells under no exogenous forces other than gravity, self-assembly of chondrocytes is driven by minimization of free energy. Reminiscent of cartilage formation in embryonic development, a cascade of cell-cell and cell-matrix interactions occur resulting in collagen VI localized around chondrocytes and collagen II throughout the neotissue. After 4 weeks, self-assembled neocartilage exhibits gross morphological, histological, biochemical, and biomechanical similarities to native cartilage (23). Studies comparing scaffold-based and scaffoldless approaches illustrate that the latter generate cartilaginous tissues with higher ECM content and mechanical properties (21, 24). Currently, scaffoldless technologies (18, 25) are undergoing clinical trials, and one could argue that scaffoldless methods should be favored.

The Challenge of Engineering Biomechanically Suitable Cartilage

One of the primary functions of bone and cartilage is to bear load. Cartilage's dense but highly hydrated matrix results in time-dependent response to loads and low friction (3). Mismatches in viscoelastic properties result in strain disparities between neocartilage and adjacent tissue, leading to tissue degradation. Cartilage also needs to withstand shear forces that exist over a large range of motion. Currently, no other materials can simultaneously match cartilage's compressive, friction, and tensile properties under large deformations and motions. In contrast, bone's biomechanical response to loading is not as time-dependent, does not involve articulation, and is more similar to traditional engineering materials, such as porous titanium, that can be fabricated to exhibit bone properties. This enables materials, such as porous hydroxyapatite, to provide initial stability and, after in vivo maturation, recapitulation of bone biomechanical properties. In contrast, evidence exists that cartilage-repair techniques, including ACI and microfracture, are unable to replicate the biomechanical properties of native tissue (11, 26).

Cartilage can experience forces up to six times the body weight and stresses approaching 10 MPa (27). Upon loading, cartilage's interstitial fluid is pressurized due to electrostatic and steric interactions that impede water flow (3) and bears the majority of load; the remainder is borne by the ECM. Clinically available cartilage-repair techniques do not recreate this structure-function relationship and, therefore, generate

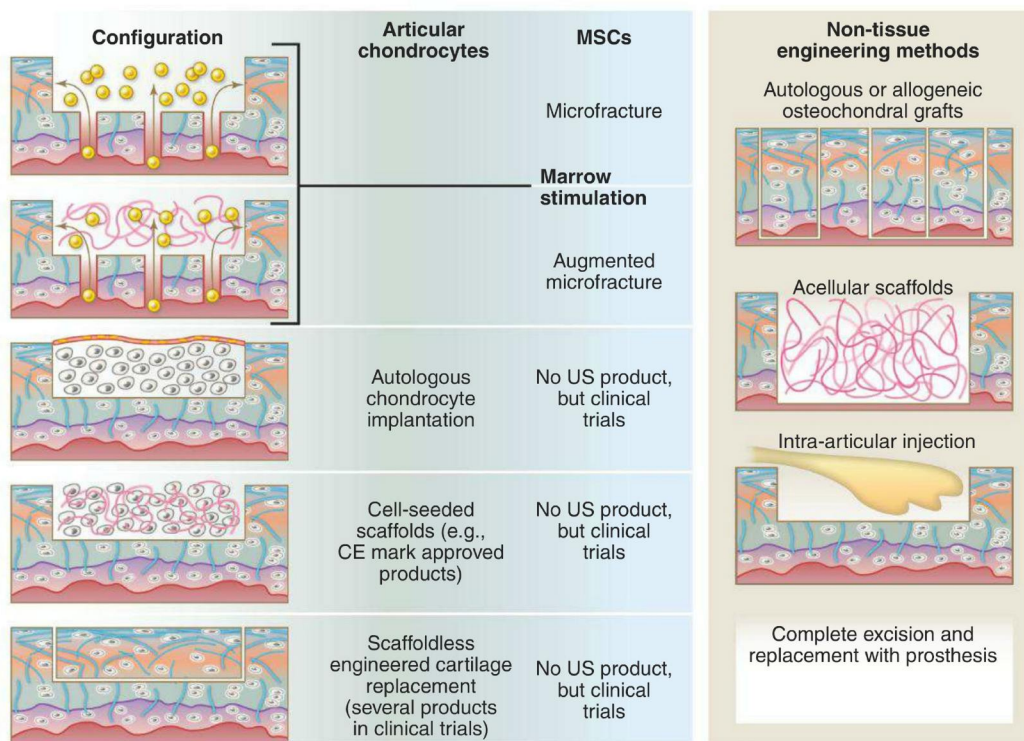


Fig. 2. Various clinical strategies regenerate cartilage using chondrocytes or MSCs. Microfracture involves subchondral bone penetration to release bone marrow that forms a stem cell-rich clot. Augmented microfracture adds a scaffold to the microfracture technique to concentrate and aid in stem cell differentiation. Acellular scaffolds are also used in full-thickness defects. Autologous chondrocyte implantation involves harvest of the patient's chondrocytes: The cells are expanded in vitro and then placed under a collagen membrane sutured over the defect site. Advancement of this technique involves seeding chondrocytes onto a scaffold and culturing in vitro before implantation. Scaffoldless engineered cartilage formed in vitro with chondrocytes is also used with two products currently undergoing clinical trials. In the aforementioned strategies, MSCs can be used instead of chondrocytes; however, products based on these technologies are at earlier stages of development. Osteochondral grafts taken either from lighter-load-bearing regions of the patient's own joint or cadaveric joints are implanted to fill focal defects. Intra-articular injections (e.g., hyaluronan) reduce the symptoms of cartilage degeneration, but the effects are only temporary. Total joint replacement is the final option when cartilage damage is so extensive that no other therapies can be effective.

tissues with deficient compressive properties (9–11, 26). Numerous stimuli can enhance neocartilage's compressive properties. For example, temporally coordinated transforming growth factor- β 3 (TGF- β 3) and dynamic compression have generated neocartilage possessing compressive properties equivalent to native tissue (28). Thus, although proper compressive properties following microfracture and ACI have been difficult to obtain, tissue engineering techniques have enabled the in vitro generation of tissues that possess the compressive properties of native tissue.

Cartilage's kinetic coefficient of friction is less than 0.005, besting most man-made bearings by at least one order of magnitude. Without this low frictional property, contact shear results in considerable wear. Cartilage garners its nearly frictionless properties from a complex combination of fluid film lubrication (forming a thick fluid film between opposing surfaces), boundary lubrication (forming a thin film of sacrificial molecules), interstitial fluid pressurization (limiting normal loads on opposing ECM), and a migrating contact area (ensuring that the fluid phase bears the majority of the normal loads) (29). Cartilage can be engineered with frictional properties similar to those of native tissue (7, 30); TGF- β 1 and shear mechanical stimulation are both effective at lowering the friction coefficient (31, 32). Considering that frictional test parameters have not yet been standardized for cartilage, future studies must emphasize not only the engineering of low-friction properties, but also the development of test standards.

As with frictional properties, tensile properties are critical to the success of cartilage tissue engineering. Although minimized by low friction, cartilage experiences tensile strains (i) during articulation

from the drag between opposing surfaces, (ii) during compressive loading in areas adjacent to the vicinity of loading, and (iii) due to its propensity to swell (3). Cartilage's surface collagen is parallel to the direction of shear to optimize tensile resistance. To anchor cartilage to bone, deep-zone collagen is oriented perpendicular to the surface. This highly organized and extensively cross-linked ultrastructure and its concomitant tensile properties have been difficult to recreate in the laboratory. Nonetheless, progress has been made through the use of remodeling agents (e.g., chondroitinase), TGF- β , and mechanical stimulation for increasing engineered cartilage's tensile modulus values to more than 3.4 MPa (33–35). To reach native tissue tensile values (5 to 25 MPa) (3), greater emphases should be placed on enhancing collagen organization, maturation, cross-linking, and content—qualities that are notoriously deficient in neocartilage. Cartilage mechanical properties, not just histology or biochemistry, must be assessed as part of demonstrating efficacy, as per the U.S. Food and Drug Administration (FDA)'s relevant guidance document (36).

Is it Possible to Integrate Engineered and Native Tissue?

Integration is critical to the success of tissue replacement, as it provides stable biologic fixation, load distribution, and also the proper mechanotransduction necessary for homeostasis. Osseointegration to a variety of materials readily occurs and provides stability for metallic implants, collagen scaffolds, and porous ceramics, due to bone's high metabolism and cells, including stem cells (19, 37). Vertical integration of cartilage to underlying bone occurs to a considerable extent; however, lateral integration of cartilage to adjacent

cartilage is rarely, if ever, reported (38). This challenge is a major stumbling block to the success and commercialization of regenerative strategies and must be addressed to achieve permanent cartilage replacement.

By harnessing the healing capability of bone, cartilage can be integrated into full-thickness defects, reestablishing loading and anchoring neocartilage to underlying bone. By placing neocartilage in direct apposition with bone, a transitional area, histologically similar to the native cartilage-to-bone interface, is recreated (38). However, incidences of delamination suggest that histological appearance is not indicative of a functional interface (39). Thus, tissue engineers have recently begun to generate osteochondral constructs to ensure that a mechanically stable interface can be created (40). Upon the construct's implantation, it is expected that the osteoinductive ability of the ceramic “bone” will facilitate stable fixation. Overall, vertical integration is driven by bone and not cartilage.

Cartilage-to-cartilage integration is exceedingly difficult to achieve, because cartilage displays low metabolism and contains dense, anti-adhesive ECM (41). For example, proteins transcribed from the *PRG4* gene, contributors to cartilage's low friction, and GAGs have been shown to directly inhibit cell adhesion (42). Further reducing integration potential, surgical preparation of defects results in cell death at defect margins (43). In the clinic, vertical integration can be assessed by the use of magnetic resonance imaging, but no imaging techniques exist with sufficient resolution to inform the extent of microscopic lateral integration. Upon loading, mismatches between the biomechanical properties of the cartilage implant and native tissue result in stress concentrations diminishing

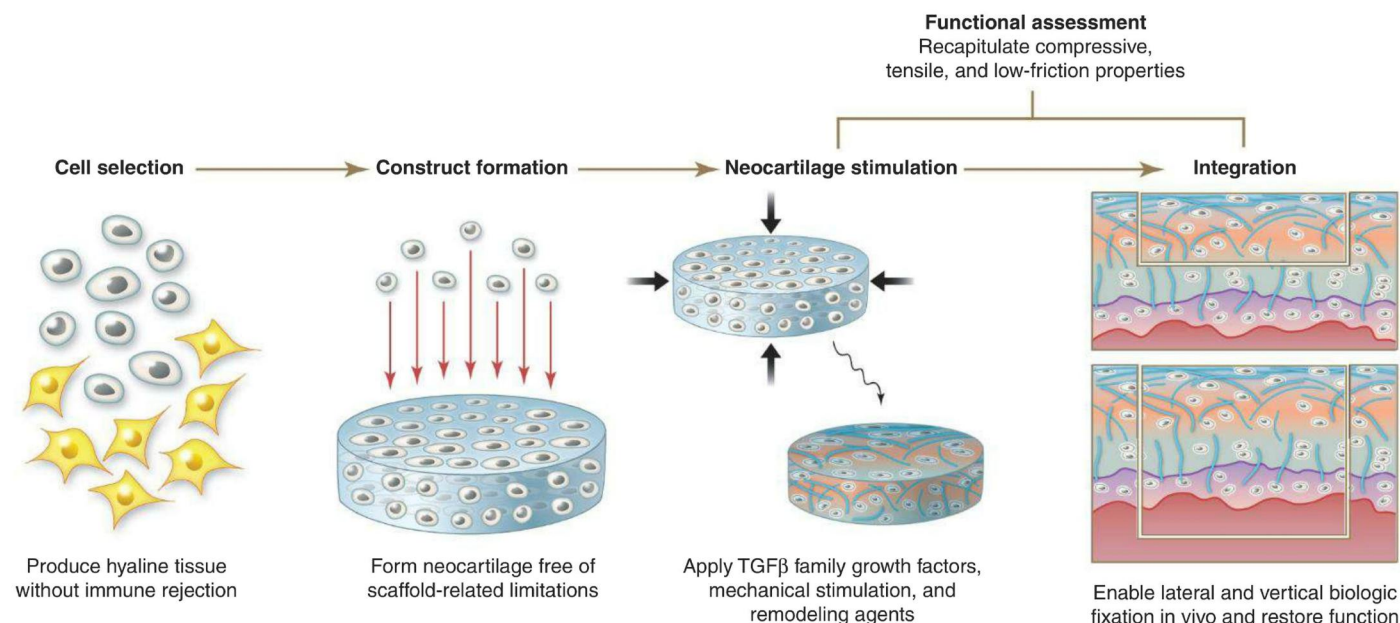


Fig. 3. Scaffoldless tissue engineering. The cell source chosen for cartilage generation, treated with appropriate culture conditions, must have the ability to produce a matrix specific to articular cartilage and must not evoke an

immune response. TGF- β family growth factors, physiologic mechanical stimulation, and matrix-remodeling enzymes have shown a large degree of promise as stimuli.

integration and damaging surrounding tissue (38). Strategies to enhance lateral integration in the laboratory include anti-apoptosis agents to mitigate cell death at the defect edge (43), matrix-degrading enzymes to decrease ECM anti-adhesive properties (41), and, more recently, scaffold functionalization to enable direct bonding to adjacent cartilage (44). Because safety and efficacy of these treatments have yet to be determined, lateral integration remains a major problem. Additionally, it is conceivable that rehabilitation protocols could be developed to enhance the biomechanical milieu of the interface and, thus, promote lateral integration.

Future of Cartilage Engineering

Scaffoldless neocartilage can now be formed with high fidelity to native tissue using expanded chondrocytes and various exogenous stimuli (Fig. 3). Strategies for in vitro vertical integration have been developed, and several candidates for lateral integration appear to be promising. Adding to existing procedures such as ACI, a plethora of new technologies is under development (table S1).

Several of these technologies directly address the hurdles of cartilage regeneration: Fibroblast growth factor-18 (FGF-18) stimulates cartilage growth and decreases cartilage degeneration scores in an osteoarthritis model (45). FGF-2 primes cells for chondrogenesis during in vitro expansion (46). Transfection of chondrocytes to express TGF- β 1 enhances cartilage formation (47). Purification using cellular molecular markers associated with high chondrogenic potential enhances the hyaline quality of cartilage formed from expanded chondrocytes (48). Emerging technologies are also harnessing the superior cartilage generating ability of juvenile chondrocytes (18) and cocultures of primary chondrocytes with MSCs (49). Scaffolds now include biphasic, osteochondral designs that may immediately bear load (50). Scaffoldless approaches also allow for the formation of constructs that can be immediately load-bearing upon implantation (25, 51). Another emerging area involves the functionalizing scaffolds with moieties, such as *N*-hydroxysuccinimide, that chemically bind to collagen (52). Stimulation during neocartilage formation using mechanical (17), anabolic (47), and, potentially, catabolic stimuli (35) may also be employed. Pathway analysis among diverse classes of stimuli allows for their strategic combination to result in synergistic interactions in cartilage formation.

For FDA approval, new cartilage therapies should show durable repair. However, toughness and hardness, both important for characterizing resistance to wear, are seldom reported, nor have technologies been developed to closely approximate these properties. In addition to mineralization, bone quality has been correlated with the degree of matrix cross-links (53), but data on cartilage cross-links in engineered or repair car-

tilages are currently absent. Associating cartilage durability with such biochemical parameters may deliver new technologies that drive the next phase of cartilage healing: durable repair that prevents the onset of osteoarthritis altogether.

Although currently the healing of cartilage defects continues to be elusive, given that emerging technologies are being validated clinically, the field is primed for an explosion of cartilage-regeneration techniques that should excite those suffering from cartilage afflictions. Furthermore, whereas osteoarthritis is currently an intractable problem, exciting new discoveries bode well for the eventual healing of a problem that afflicts a quarter of our adult population.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/338/6109/917/DC1 Table S1

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REVIEW

Printing and Prototyping of Tissues and Scaffolds

Brian Derby

New manufacturing technologies under the banner of rapid prototyping enable the fabrication of structures close in architecture to biological tissue. In their simplest form, these technologies allow the manufacture of scaffolds upon which cells can grow for later implantation into the body. A more exciting prospect is the printing and patterning in three dimensions of all the components that make up a tissue (cells and matrix materials) to generate structures analogous to tissues; this has been termed bioprinting. Such techniques have opened new areas of research in tissue engineering and regenerative medicine.

The development of implantable medical devices and organ transplantation has radically changed the scope of medical inter-

vention to deal with chronic medical problems and potential end-of-life situations. However, issues of device failure, the limited supply of donor

organs, and the need for immunosuppressants are ongoing problems. The broad areas of tissue engineering and regenerative medicine seek to avoid some of these problems through the augmentation of the healing process or the fabrication of autologous tissue grafts that will not cause an immune response in the host. It has been suggested that we will be able to build complete organs from scratch, and the term “organ printing” has been coined and used by the popular press. Although such a goal is unlikely to be attainable in the near future, printing and related technologies are of current use in the areas of tissue engineering and regenerative medicine.

An important concept in tissue engineering is the scaffold, a three-dimensional (3D), highly porous substrate. Cells donated by the patient are expanded in culture and are then transferred to the scaffold. The scaffold provides a surface on which cells adhere, thrive, multiply, and generate the extracellular matrix (ECM) of structural and functional proteins and saccharides that make up living tissue. Both the scaffold material composition and its internal architecture (dimensions of the struts, walls, pores, or channels) control the behavior and well-being of the cells seeded inside.

Within the body, cells must be very close to the capillary network that supplies oxygen and nutrients. Similarly, within a tissue scaffold, all cells must be supplied with the means to maintain life, and this is achieved initially by providing a highly porous open structure to allow the uninterrupted flow and access of culture media in a bioreactor. In many ways, the engineering component of tissue engineering lies in the design and manufacture of the scaffold. Hutmacher (1) gave an early analysis of the topological requirements of a suitable scaffold material and reviewed the manufacturing routes that could be used to achieve the desired structure. Traditionally, porous scaffolds have been made by a number of routes that result in a foam-like internal structure with a random architecture and a limited control of scale. However, the development of rapid prototyping techniques since the 1980s has enabled fabrication of fine-scale internal porous structures with the desired complexity, allowing a true engineering of the scaffold (1, 2).

Rapid prototyping methods, sometimes referred to as 3D manufacturing or solid freeform manufacture, produce complex objects from a 3D design file by decomposing the object's shape into a series of parallel slices. The shape is then fabricated by reproducing these slices a layer at a time, building up the structure. The philosophy of the method is to create objects by adding material layer-by-layer; hence, it is now generally referred to as additive manufacture (AM) to dis-

tinguish it from conventional machining, which removes material in a subtractive manner. With design files now held in digital format as standard, it is relatively easy to convert an arbitrary object into the slices required by AM fabrication tools. These slice design files are used to generate solid layers using a range of manufacturing techniques, including selective polymerization, selective sintering/melting, building solids through the laying down of viscous threads, and 3D inkjet printing.

These techniques have been used to manufacture laboratory components (3) or cell micro-wells (4) to order. The hardware cost for these fabrication tools has also decreased, and open-source equipment is now available from a number of groups (5, 6). Other workers have “hacked” commercial desktop printers to fabricate 2D and 3D objects from engineering and biological materials (7–10). These techniques and their applications in tissue engineering have been reviewed extensively (1, 11). Early scaffolds were fabricated by AM from a single biocompatible material. It is now possible to engineer materials that contain biomimetic components to control the cellular environment (12, 13). The scaffold should be a temporary feature and disappear, through dissolution or degradation, as the cells produce the ECM that defines the tissue. The materials that make up the scaffold must be designed to fulfill this function and be compatible with the fabrication route.

Another approach to tissue engineering has been proposed that does not require a solid scaffold structure. Some workers have championed what has been identified as “bottom-up” or “scaffold-free” tissue engineering (14), which starts from the belief that tissue is a cell-generated material, and thus the direct manipulation and control of cells is of greater importance than the provision of structural, mechanical, and chemical cues via the intermediary of a scaffold. In such an approach, cells are formed into clusters (15), aggregates (16, 17), or 2D sheets (18). These are then manipulated or positioned into 3D cellular constructs by using clusters as “bricks” or sheets as laminates. This methodology has a number of potential advantages over scaffolds:

- Because no scaffold is present, there are no problems of material or degradation product compatibility.
- Cells are cultured in conditions more similar to the 3D environment of the body, allowing better intercellular communication.

- Clusters made of cells that serve complex functions in organs and are very sensitive to the local environment (e.g., hepatocytes) are less likely to redifferentiate and lose function (15).

However, there are other aspects of tissue engineering where the presence of a scaffold may be beneficial:

- Scaffolds provide mechanical strength while ECM is being generated.
- Assembly of large cellular constructs from sheets or clusters is limited by transport of oxygen, nutrients, and waste products until a vascular system develops.

Thus, a hybrid approach intermediate to the scaffold-directed approach (seeded cells) and scaffold-free approach (organized cellular sub-components) is likely to be developed. This is certainly the case where complex organ-like structures are required; in these instances, the strategy of “let the cells decide” used in the scaffold-free approach will probably need to be assisted by the use of biochemical cues to direct the desired outcome.

The concept of bioprinting, which is essentially an extension of the philosophy that uses AM methods to build complex scaffold structures, can be thought of as a combination of (i) different types of cells in defined locations, (ii)

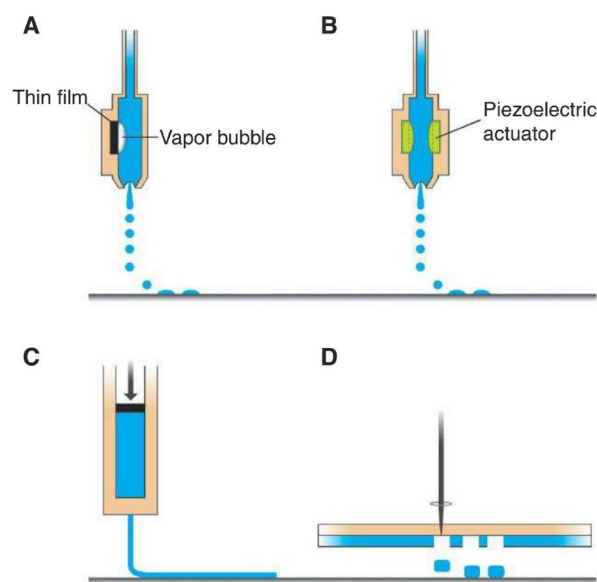


Fig. 1. In additive manufacture (AM), a rapid prototyping technique used in bioprinting applications, solids are produced through the sequential deposition of solid layers or slices. (A and B) Inkjet printing accurately positions drops, which may contain cells in suspension, in the volume range 1 to 100 pl. Drops are generated either by heating and vaporizing the ink so that a bubble forces out a liquid drop (A) or by mechanical actuation (B). (C) Microextrusion or filament plotting deposits a continuous thread of material to build up a layer. (D) With laser forward transfer, material is initially deposited on a transparent ribbon in gel form. A pulsed laser beam vaporizes a small quantity of material; this ejects the remainder of the material illuminated, which is transferred to the substrate across a small air gap. [Panels A and B reproduced with permission from (24)]

supporting matrix or scaffolds (if required), and (iii) biochemical cues to control behavior. A co-deposition strategy involving chosen combinations of these three elements can be achieved with the use of printing or AM tools to define their 3D arrangement—either topologically or by providing channels for nutrient access (19). Although bioprinting has its origins in the area of tissue engineering and is sometimes described as organ printing (10), there are other application areas where a printed or artificially fabricated tissue analog structure is useful, including cell-based sensors (20), drug/toxicity screening (21), and tissue or tumor models (22).

A key feature of bioprinting is that the deposition process must be cytocompatible, as it requires the dispensing of cell-containing media. This reduces the range of AM techniques that are feasible because of the need to work in an aqueous or aqueous-gel environment at temperatures from room temperature to 38°C. Hence, most bioprinting work reported in the literature has used filament extrusion, inkjet printing, or laser forward transfer (Fig. 1).

Controlling 3D Architectures

In manufacturing engineering, AM frees the designer from the constraints of molds and dies, thereby drastically reducing the cost of producing a single, one-off design. It also allows the exploration of design variables. In tissue engineering, this enables the easy variation of the internal architecture of a scaffold, allowing the manufacture of a range of structures to allow optimization, or libraries of structures that can be used in rapid-throughput screening for a given application. Melchels *et al.* (23) used stereolithography (a well-established AM method) to produce a range of scaffold designs with different internal architectures and porosity (Fig. 2) from a biocompatible and degradable polymer. By altering the internal architecture, it is possible to vary the specific surface area, scaffold mechanical response to external stress, and flow resistance in a bioreactor. Thus, variations in complex internal scaffold architecture can be easily explored as part of an experimental study. Another advantage of architectural control of a porous structure is the ability to better select and control mechanical properties such as stiffness and strength. Conventional tissue scaffolds are fabricated through the foaming of fluid precursors or leaching of pore-generating particles around which a scaffold

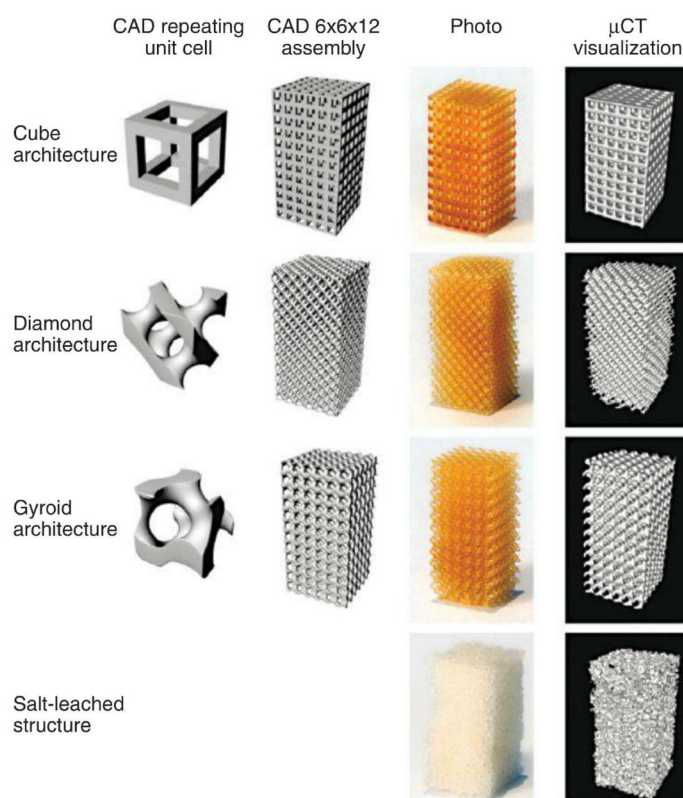


Fig. 2. Recent advances in AM technology, tomographic reconstruction, and numerical modeling methods have allowed workers to design a range of complex internal scaffold architectures with a range of length scales. These can be fabricated with a minimum feature length of <100 μm and the resulting structures inspected for perfection. This figure shows the design file, image of fabricated scaffold, and 3D tomographic reconstruction for three different designed scaffold architectures and a salt-leached structure formed by a random foaming process. [Reproduced with permission from (24)]

fold has been formed (1); this produces a fairly random structure when compared with a scaffold fabricated through AM methods (Fig. 2).

With 3D manufacture, rapid-throughput screening of structural variation can be difficult because each structure requires a finite volume of study. However, considerable progress can be made through the use of highly structured surfaces (so-called “2½D” objects), where micromanufacturing methods can be used to provide a large range of structures with relatively high aspect ratios by varying a small number of structural units or building blocks. Unadkat *et al.* have demonstrated a high-throughput method of studying the influence of surface topology on the fate of human mesenchymal stem cells (24). They used conventional silicon lithographic methods to form a master stamp to pattern a thermoplastic biomaterial, poly(lactic acid). Because the variation in surface topology was spatially correlated, they were able to use optical probes and image analysis techniques to correlate cell response with local topology. An example of their “topo-chip” is illustrated in Fig. 3.

As well as allowing the exploration of experimental variables in scaffold design and providing controlled features to study cell interactions, AM-produced tissue scaffolds have recently progressed from the laboratory toward clinical application. Two recent studies have demonstrated substantial progress. Reichert *et al.* carried out a large-scale trial using a sheep model for hard-tissue regeneration in long bones (25). This study compared the performance of a tissue engineering solution, using polycaprolactone-hydroxyapatite composite scaffolds fabricated by a filament deposition method (fused deposition modeling), to that of an autologous graft. The implant-tissue integration was assessed biomechanically and by x-ray computed tomography; the behavior of the AM-produced implant was equivalent to that of the autologous graft. Probst *et al.* have reported the successful outcome of a trial clinical animal model translation study that used a designed porous scaffold fabricated by AM (26). It is highly likely that AM scaffolds will be used clinically in hard-tissue reconstructive surgery in the near future, notwithstanding the considerable regulatory hurdles that still need to be overcome.

A key problem in the manufacture of scaffold structures by AM methods is the need to fabricate internal cavities via a layer-by-layer additive process. Such cavities or pore networks are required to assist nutrient transport in a bioreactor and to provide a framework to encourage neovascularization as ECM is deposited. Self-evidently it is difficult to deposit a material above an empty cavity, and this problem is overcome through the use of either temporary support structures or sacrificial materials that are leached away after manufacture to form cavities (2, 27). The use of leaching to form cavities is limited by the need for cytocompatible solvents and leaching products; recent work by Miller *et al.* has proposed an elegant solution to this problem by developing a soluble carbohydrate glass formulation (28) that can be readily dispensed by filament microextrusion to form a network that can be leached with water to form a series of interconnected fine cylindrical channels (Fig. 4).

Bioprinting

Bioprinting has been defined as the positioning of biochemicals, biological materials, and living cells (19). It is distinct from AM technologies

used to fabricate scaffolds because it allows the deposition of different materials via the same equipment (e.g., the fabrication of structural materials and the simultaneous or sequential deposition of cells) (4). By analogy with color printing, we can use the technology to print discrete patches of different materials (or gradients and transitions between materials) to explore their influence in more detail. Printing can be conveniently divided into analog and digital printing. Analog printing is normally the direct transfer of an image by some contact process onto a substrate. Common high-resolution contact printing technologies used for bioprinting applications include microcontact printing and dip-pen lithography. These have both been used extensively to pattern surfaces with biochemicals and adhesion modifiers (29, 30), chiefly to study how surface features affect the behavior of cells. Both technologies sacrifice speed of application to achieve very high spatial resolution, typically $<1\ \mu\text{m}$, and neither can be used to deposit cells. This review is limited to computer-controlled and digital printing processes. In digital printing, the image is converted into pixels and the print consists of a digital pixel on/off application of material. Many forms of digital printing are contactless; the most common is inkjet printing. Another digital printing technology, laser forward transfer, has been used extensively for bioprinting applications. The most important computer-controlled deposition process is plotting or microextrusion.

Printing Surfaces and Structures to Control the Distribution of Cells

Inkjet printing is ideally suited to the deposition of biological materials because it is designed for the generation and precise positioning of picoliter (pL) volumes of fluid (31); these liquid drops can be aqueous solutions and can thus be used for the deposition of most biological species (19). Inkjet printing was used as a tool for the deposition of cells and biological materials in 1988 by Klebe, who used a Hewlett-Packard desktop printer to deposit collagen and fibronectin suspensions as well as cells to form 3D simple tissue analog structures (9). This study used an unmodified printer and simply replaced the graphics ink in a cartridge, after appropriate cleaning, with a biological fluid. Desktop printers with unmodified ink delivery but with a new ink formulation have been used to deposit functional inorganic materials (7, 8) as well as cells and biological

materials (32), illustrating the versatility of this fabrication tool.

The surface resolution attainable by inkjet printing is controlled by the size of a printed drop in flight and its contact angle on the surface after impact (33). The smallest drops available through inkjet printing are $\sim 1\ \text{pL}$ in volume (radius $\approx 6.2\ \mu\text{m}$) and thus, on mildly hydrophilic surfaces, they have a minimum feature dimension of $>10\ \mu\text{m}$, similar to that of whole cells. Using electrohydrodynamic drop generation, it is possible to produce linear features $\sim 2\ \mu\text{m}$ in width (34). Early work featured the use of surface patterning to produce large areas on a surface to selectively control cell adhesion (35).

One advantage of inkjet printing over analog printing methods is that it is possible to engineer variation in surface concentration through overprinting at different drop densities (Fig. 5). This has been exploited by Campbell *et al.*, who demonstrated that the response of cells could be directly correlated the surface concentration of printed hormones (36). They were also able to show that surface concentrations of growth factors are able to control stem cell fate and that different patterns could be used to differentiate cell fate in the same culture dish (37). These

studies used flat polystyrene surfaces for printing and subsequent cell culture. In tissue, cells experience a 3D environment, so the bioprinting results may not be truly representative. Thus far, fabricating 3D distributions of growth factors in matrices to probe cell behavior has not been achieved. However, Campbell and colleagues have attempted to address some of these issues by using nanofibrous scaffolds onto which growth factors are printed (38).

Direct Cell Printing

If the goal of bioprinting is to reproduce tissue structures, it must be capable of fabricating complex heterogeneous architectures—either by positioning different cell types in desired locations, or by inducing progenitor cells to differentiate into the desired type in specific locations. Achieving these tissue analog or tissue progenitor structures requires a 3D fabrication facility with multiple material capability in order to fabricate a temporary structural support with an open structure to allow the flow of nutrients, along with the ability to position cells and biochemicals with controlled delivery (39). Considering that our ability to fabricate and characterize simple single-material scaffolds is relative-

ly recent, the target of a printed tissue is highly ambitious. Three main technologies are used for deposition and patterning with cells: inkjet printing (9, 32), microextrusion or filament plotting (9, 40), and laser forward transfer (41). Similar technologies have also been used to transport and place clusters or aggregates of cells (10). These techniques have comparable levels of maximum attainable spatial resolution, $\sim 100\ \mu\text{m}$. Much higher resolution can be attained using techniques such as electrohydrodynamic jetting (34), which has been demonstrated with cell suspensions (42). However, given that typical cell densities used during culture are $\sim 10^7\ \text{cells/mL}$, an individual volume of $100\ \text{pL}$ (drop radius $\approx 70\ \mu\text{m}$) will contain on average a single cell; this limits the effective accuracy of deposition for cell placement.

One concern is the level of stress that cells experience during deposition processes. Each method for fabricating cell-containing structures has different requirements for the material surrounding the cell. Inkjet printing requires a low-viscosity environment to allow efficient drop ejection (31), microextrusion methods have a very wide range of fluid properties that are compatible with the process but offer a lower spatial resolution, and laser forward transfer

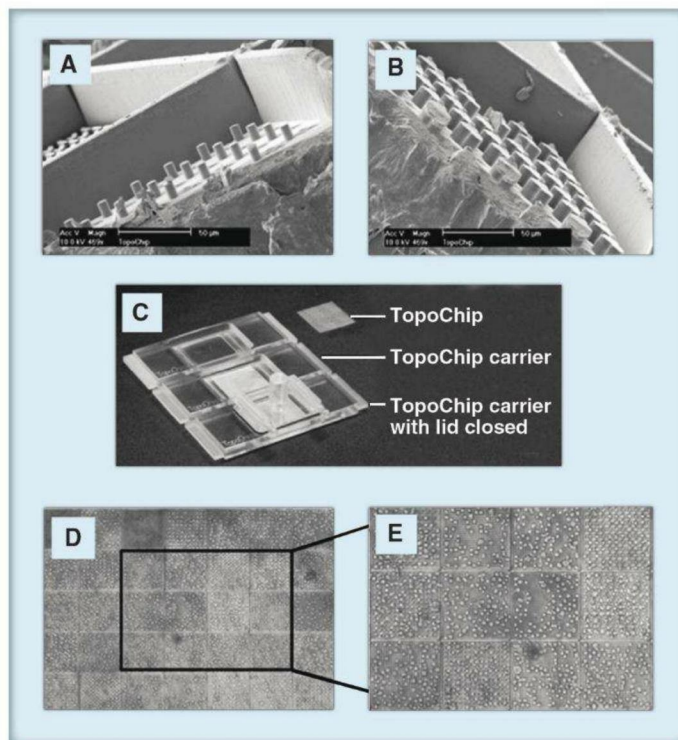


Fig. 3. Example of a “topochip”—a method of producing a variation in surface structure that can be identified by spatial correlation. (A and B) Examples of different surface topologies at different locations on the surface (scale bar, $50\ \mu\text{m}$). (C) TopoChip (with dimensions $2\ \text{cm} \times 2\ \text{cm}$) and its carrier system. (D and E) Local cell behavior can be identified and correlated to the known position of each surface topology unit ($280 \times 280\ \mu\text{m}$). [Reproduced with permission from (25)]

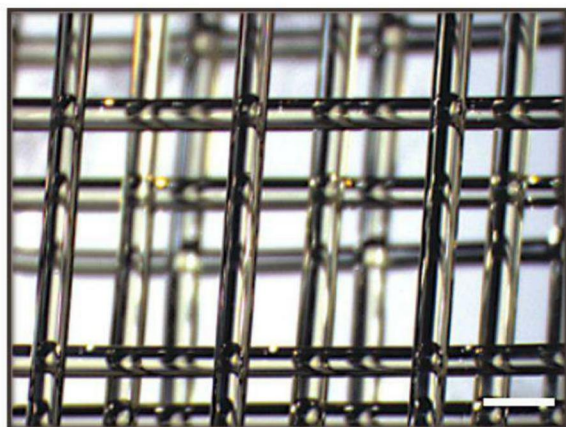


Fig. 4. Three-dimensional lattice fabricated from a carbohydrate glass structure using a filament extrusion AM technique. A photopolymerizable cell suspension is cast around this structure, which is subsequently removed by leaching to provide a vascular network. Scale bar, 1 mm. [Reproduced with permission from (29)]

requires the cells to be immobilized in a gel (41). Early reports on inkjet printing of cells reported poor viability after deposition (32); however, subsequent work found cell survival rates consistent with those of unprinted controls (i.e., survival >95%) with appropriate choice of printing conditions (43–45), which suggests that the precise formulation of the ink is important. It is noteworthy that Cui *et al.* (45) found that after printing, the cell membrane had a transient increase in density of nanopores; this allowed a greater transfection rate than with unprinted cells and may indicate the mechanism of cell damage that occurs during printing. Studies of damage to cells during microextrusion experiments have been less systematic. Chang and

Sun have reported cell survival rates in the range of 40 to 80% after extrusion of HEPG2 cells, with the survival rate decreasing with increasing extrusion pressure (46). This trend of increasing mechanical stress reducing survival after deposition is consistent with Saunders' data for cell survival after inkjet printing, where an increase in the actuating voltage used to generate the drops led to a decrease in cell survival (44). The same trend is seen with laser cell transfer, where a greater amount of optical energy is seen to decrease cell survival rates after deposition (47).

Bioprinting, which began as a concept for cell manipulation (9) and is now a viable technique for patterning with cells (32, 40, 46), has been combined with a number of biological materials to directly produce hybrid cell-containing structures. D'Lima *et al.* used an aqueous solution of poly(ethylene glycol) dimethacrylate that contained chondrocytes in suspension and printed this into a model osteochondral defect before using a photolytic cross-linker to form a hybrid cell-containing hydrogel (48). After a number of days in culture, the printed structure appeared to have integrated into the surrounding tissue (Fig. 6), demonstrating the feasibility of this approach. Although this study demonstrates great promise, the general use of photoinitiated cross-linking of cell suspensions must address the potential cytotoxicity of the photoinitiator. Williams *et al.* carried out a systematic study of the influence of a number of different photoinitiators on cell populations and found that the relative toxicity of each chemical studied depended on cell type (49); hence, great care must be taken in selecting appropriate polymer-photoinitiator combinations in the presence of cells.

Prospects for the Future

We are still a long way from organ printing. Although current deposition and fabrication technologies allow us to build structures that are analogous to tissue in their composition, the development of fully functioning tissue is a much greater step. The use of AM to produce scaffold structures, mainly for bone tissue engineering, is now almost routine but is still only possible with a limited set of biomaterials. The use of AM-fabricated scaffolds appears to be making important steps toward translation (25, 26), and for hard-tissue applications, the barriers are now regulatory rather than scientific or technical. The prospects are less good for soft connective-tissue applications where more compliant materials (e.g., hydrogels) are required. Here the main technical challenges are to find suitable biomaterials with appropriate mechanical properties that can be deposited using AM methods. The challenge here is in the field of developing new biomaterials. To date, fine-detailed hydrogel or biological material scaffolds have been fabricated using

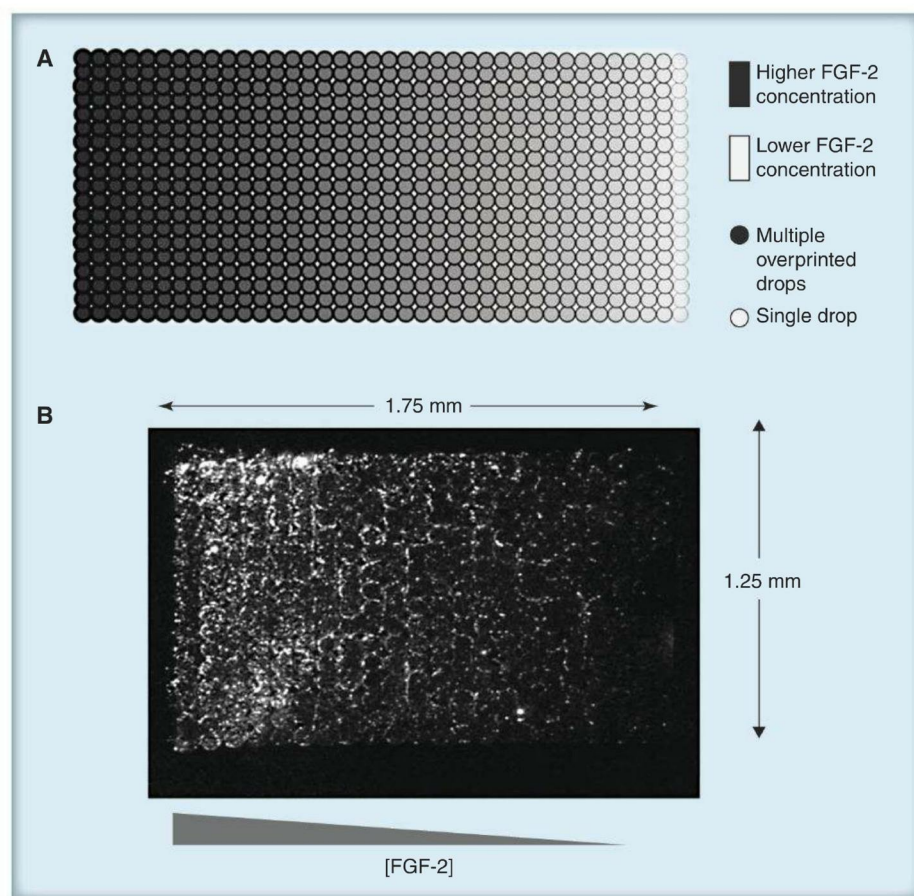


Fig. 5. Illustration of how digital printing methods can be used to print spatially defined concentration gradients of biochemicals. (A) Schematic showing how gradients of fibroblast growth factor-2 (FGF-2) are printed with different surface concentrations formed by overprinting multiple drops in the same location on the surface. (B) Example of an FGF-2 gradient prepared using sequential overprinting of biotinylated FGF-2, which was labeled with streptavidin conjugate quantum dots. [Reproduced with permission from (36)]

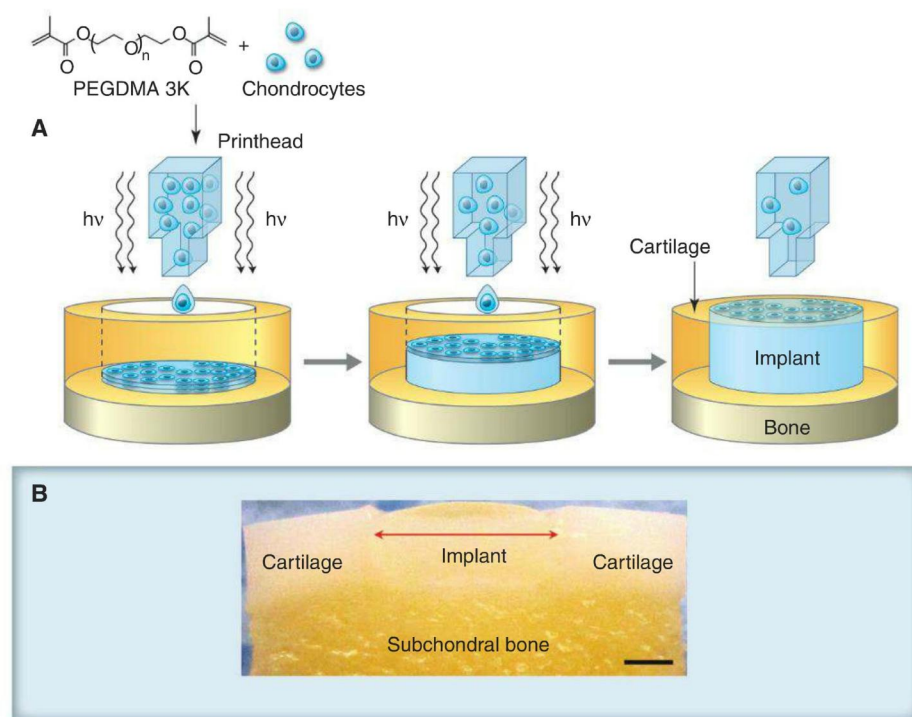


Fig. 6. (A) Schematic of bioprinting a cartilage analog structure, combining inkjet printing with a poly(ethylene glycol) dimethacrylate (PEGDMA) solution containing cells in suspension with a simultaneous photopolymerization process. (B) Light microscopy image of cell-containing polyethylene hydrogel printed into a defect formed in an osteochondral plug (scale bar, 2 mm). After culture, the cells within the printed material express ECM similar to those in the adjacent tissue. [Reproduced with permission from (48)]

indirect methods, where a sacrificial mold is built using AM (28, 29).

Bioprinting offers promise for the fabrication of structures modeled on tissue architectures. The majority of published work to date is at a relatively low level of technological readiness and has used a very limited range of materials: sodium alginate, modified diblock copolymers, and photocured acrylates. The range of available materials is severely constrained by the need to develop cytocompatible gelation mechanisms that can be delivered by AM methods using fluid delivery (inkjet and filament extrusion) and can produce a cell-containing matrix with an appropriate range of mechanical properties. There is also still considerable uncertainty concerning the level of cell damage that occurs during cell deposition by all bioprinting methods. It is clear that much further work will be needed in this area before regulatory approval can be obtained for translational studies. What is more likely is that we will use these tissue analog structures for applications such as toxicity screening and drug testing (21, 39). One interesting development is the use of microfabrication technology to construct tumor models, allowing variation in physiological conditions in vitro (50).

For any new technology to have an impact in scaffold manufacture or tissue printing, further consideration of generic problems must

also be considered. The concept of a scaffold requires an understanding of the behavior of an implanted structure within the body and its interactions at a cellular and tissue scale. The rate of scaffold degradation and ECM formation must be both understood and controllable. However, AM methods provide an important advance in scaffold design because many of the parameters of architecture that control the scaffold's physical, mechanical, and degradation properties are adjustable within fairly broad limits. Thus, as discussed in detail by Melchels (24), it is now possible to model the influence of tissue architecture and validate the model by experiments on a range of structures. A challenge here is to fully ascertain whether designed scaffold structures are reproduced by AM techniques, and the recent increase in availability of computed tomography imaging will aid this effort. Tomography will again assist in monitoring degradation in vitro, and possibly functional nuclear magnetic resonance may be useful in imaging fluid flows and degradation of scaffolds in vivo. Predictive modeling or validation of tissue engineering solutions is likely to be the area of immediate impact for these new manufacturing technologies.

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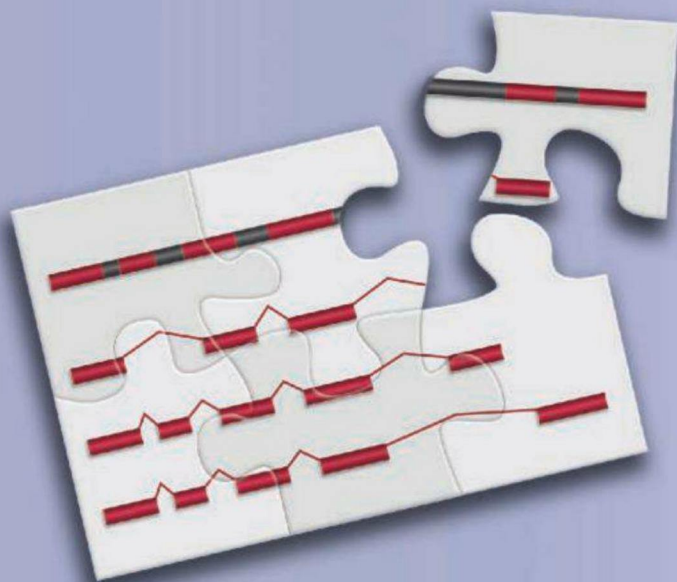
WEBINAR

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Electrically, Chemically, and Photonically Powered Torsional and Tensile Actuation of Hybrid Carbon Nanotube Yarn Muscles

Márcio D. Lima,^{1*} Na Li,^{1,2*} Mônica Jung de Andrade,¹ Shaoli Fang,¹ Jiyoung Oh,¹ Geoffrey M. Spinks,³ Mikhail E. Kozlov,¹ Carter S. Haines,¹ Dongseok Suh,¹ Javad Foroughi,³ Seon Jeong Kim,⁴ Yongsheng Chen,² Taylor Ware,¹ Min Kyoon Shin,⁴ Leonardo D. Machado,⁵ Alexandre F. Fonseca,⁶ John D. W. Madden,⁷ Walter E. Voit,¹ Douglas S. Galvão,⁵ Ray H. Baughman^{1†}

Artificial muscles are of practical interest, but few types have been commercially exploited. Typical problems include slow response, low strain and force generation, short cycle life, use of electrolytes, and low energy efficiency. We have designed guest-filled, twist-spun carbon nanotube yarns as electrolyte-free muscles that provide fast, high-force, large-stroke torsional and tensile actuation. More than a million torsional and tensile actuation cycles are demonstrated, wherein a muscle spins a rotor at an average 11,500 revolutions/minute or delivers 3% tensile contraction at 1200 cycles/minute. Electrical, chemical, or photonic excitation of hybrid yarns changes guest dimensions and generates torsional rotation and contraction of the yarn host. Demonstrations include torsional motors, contractile muscles, and sensors that capture the energy of the sensing process to mechanically actuate.

The concept of deploying strong carbon nanotube yarns as actuators has produced both electrochemically and thermally powered yarn muscles. The performance of electrochemically powered yarn muscles (1, 2) is adversely affected for most applications by the need for electrolyte, counter electrode, and device packaging, which add much more to actuator weight and volume than the actuating electrode. The electrolyte also limits operating temperature and voltage, as well as actuation rate. Previous work has demonstrated use of polymer-filled nontwisted carbon nanotube yarns as thermally powered shape memory actuators, but reversible actuation was not achieved (3). Dispersed carbon nanotubes and nanotube sheets have been used for electrically heating thermally actuating materials to produce cantilever deflections (4–6).

Here we demonstrate large-stroke, high-power, and high-work-capacity yarn muscles that provide millions of cycles and avoid the need for electrolyte or special packaging. Torsional and tensile actuation of these hybrid muscles results from dimensional changes of a yarn guest. The twist-spun nanotubes confine this actuating guest

in both solid and molten states and provide the mechanical strength and helical geometry enabling large-stroke torsional and tensile actuation. Reversible actuation is powered electrically, photonically, or by chemical absorption and desorption.

Yarn fabrication and structure. Nanotubes drawn from a carbon multiwall nanotube (MWNT)

forest are twist-spun into a yarn (7–14). The utilized forests are ~350 μm high and consist of MWNTs that have an outer diameter of ~9 nm, contain about six walls, and form large bundles. Symmetric twist insertion during sheet draw from a forest or into a predrawn nanotube sheet (suspended between either a forest and one rigid support or two rigid supports) provides the two investigated helical yarn structures (Fermat scrolls for the former cases and dual-Archimedean scrolls for the latter) (15, 16) illustrated in Fig. 1, H and I. The bias angle (the angle between the yarn length and nanotube directions) for the simpler Fermat yarn is

$$\alpha = \tan^{-1}(2\pi rT) \quad (1)$$

where r is the distance from yarn center and T is the inserted twist in turns per yarn length. The measured bias angle is that for the yarn surface, where r equals the yarn radius. Overtwisting these MWNT yarns, as for ordinary textile yarns, rubber bands, and DNA molecules, causes coiling, which is called writhe (17–20). This coiling, as well as coiling in plied yarn, will be used to dramatically amplify tensile stroke and work capabilities compared with those for uncoiled yarn (21).

Methods for incorporating guest actuating material into the host yarn include melt and solution infiltration (which can be followed by in situ polymerization) and biscrolling, in which the guest is deposited on a MWNT sheet before twist insertion.

Nanotube muscle chirality and tethering. Yarn volume during actuation for nonplied hybrid

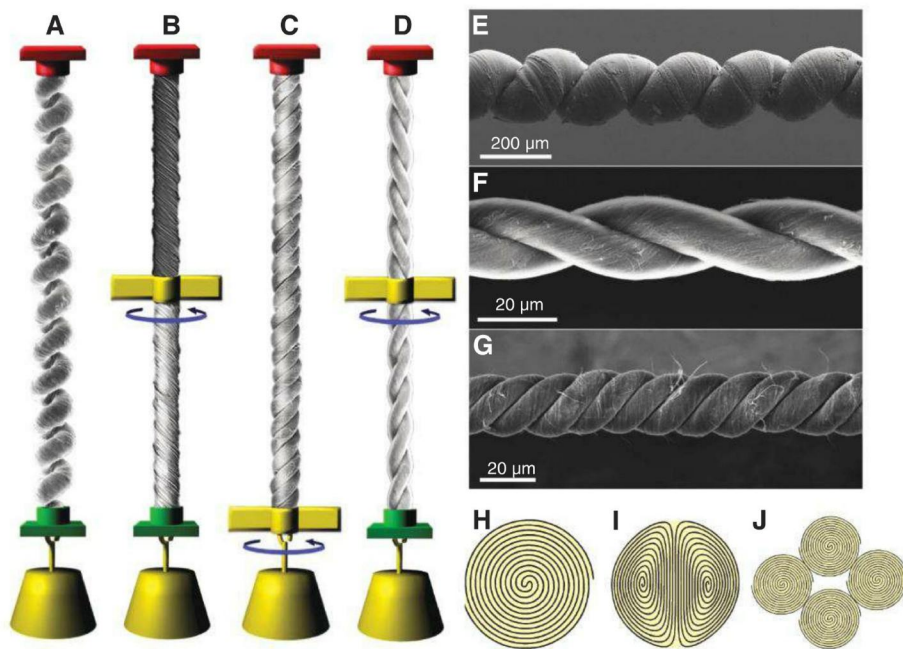


Fig. 1. Muscle configurations and yarn structures for tensile and torsional actuation. Tensile load and paddle positions for (A) a two-end-tethered, fully infiltrated homochiral yarn; (B) a two-end-tethered, bottom-half-infiltrated homochiral yarn; (C) a one-end-tethered, fully infiltrated homochiral yarn; and (D) a two-end-tethered, fully infiltrated heterochiral yarn. The depicted yarns are coiled, noncoiled, four-ply, and two-ply, respectively. Arrows indicate the observed direction of paddle rotation during thermal actuation. Red and green yarn-end attachments are tethers, meaning they prohibit end rotation; red attachments also prohibit translational displacement. SEM micrographs of (E) a fully infiltrated homochiral coiled yarn, (F) a neat two-ply yarn, and (G) a neat four-ply yarn. Illustration of ideal cross sections for (H) Fermat, (I) dual-Archimedean, and (J) infiltrated four-ply Fermat yarns.

¹The Alan G. MacDiarmid NanoTech Institute, University of Texas at Dallas, Richardson, TX 75083, USA. ²Centre of Nano-scale Science and Technology, Institute of Polymer Chemistry, College of Chemistry, Nankai University, Tianjin 300071, China. ³Intelligent Polymer Research Institute, Australian Research Council Centre of Excellence for Electromaterials Science, University of Wollongong, Wollongong, NSW 2522, Australia. ⁴Center for Bio-Artificial Muscle and Department of Biomedical Engineering, Hanyang University, Seoul 133-791, South Korea. ⁵Applied Physics Department, State University of Campinas, Campinas, SP, 13081-970, Brazil. ⁶Faculdade de Ciências, Universidade Estadual Paulista, Baurur, SP, 17033-360, Brazil. ⁷Department of Electrical and Computer Engineering, University of British Columbia, Vancouver, BC V6T 1Z4, Canada.

*These authors contributed equally to this work.

†To whom correspondence should be addressed: E-mail: ray.baughman@utdallas.edu

yarns (i.e., yarns that are not wrapped around another yarn) causes tensile contraction and untwist. Since the untwisting of a nonplied actuating yarn segment causes elongation that partially cancels yarn contraction, maximizing tensile contraction and torsional actuation generally require different configurations.

We predict configurations that optimize either torsional or tensile actuation for yarns, where the only variables are whether guest infiltration is along the entire yarn or one-half its length, whether the yarn is homochiral (one chirality) or heterochiral (with equal length segments having opposite chirality), and whether the yarn is nonplied or plied. Using opposite chirality, nonplied yarn segments (designated S and Z), with a paddle at their interconnection (Fig. 1D), maximizes initial torque on the paddle, because these segments operate additively to provide rotation. For the same yarn, the one-end-tethered configuration of Fig. 1C provides twice the torsional rotation of the Fig. 1D configuration, but one-half the initial torque, so both configurations provide equal torsional work capacity. Actuation of one segment in a two-end-tethered homochiral yarn (Fig. 1B) generates smaller rotation than for the heterochiral yarn of Fig. 1D because of the energetic cost of twisting the unactuated yarn as the actuating yarn untwists. As with the Fig. 1C configuration, the Fig. 1D configuration with nonplied yarn does not provide reversible actuation unless internally constrained by a solid guest, to prevent S twist from canceling Z twist in the other yarn segment.

Yarn untwist is prohibited in Fig. 1A unless symmetry is broken for energetic reasons to provide yarn segments with opposite changes in twist, while untwist of the actuating segment is compensated by up-twist of the nonactuated segment in Fig. 1B, so these actuator configurations can optimize tensile contraction per length for a nonplied ac-

tuated yarn. Because the entire yarn untwists for the Fig. 1, C and D, configurations during actuation when the yarn is nonplied, they do not provide optimized tensile contraction.

Thermally, electrothermally, and photothermally powered actuation. Thermal actuation of hybrid yarn muscles is largely driven by volume expansion of the yarn guest. Paraffin waxes are used as prototypical guests because of high thermal stability; the tunability of transition widths and temperatures; the large volume changes associated with phase transitions and thermal expansion; and their ability to wet carbon nanotube yarns. Such waxes have been long investigated and commercially deployed as thermally or electrothermally powered actuators (22). By confining the actuating wax in the nanosized pores of a MWNT yarn (fig. S3), the goal is to avoid conventional hydraulic and external heating systems and directly use a muscle-like geometry, where high surface/volume and thermal and electrical conductivities enhance response rate and a helical geometry enables both torsional rotation and tensile contraction. Results described are for a commercial wax ("Aldrich wax," Sigma Aldrich 411671), which fully melts at $\sim 83^\circ\text{C}$, increases volume $\sim 20\%$ between 30° and 90°C , and expands an additional $\sim 10\%$ between 90° and 210°C (fig. S5).

Tensile contraction versus temperature for coiled dual-Archimedean yarn, before and after wax infiltration, is compared in Fig. 2A with corresponding data (figure inset) for noncoiled Fermat yarn. Wax infiltration greatly enhanced tensile contraction for all yarns, as did yarn coiling. Despite a difference in the load dependence of actuation, similar tensile strokes were obtained for noncoiled, Fermat, and dual-Archimedean yarns having similar diameter and twist angle (fig. S2). Heating the neat coiled yarn from ambient to incandescent

temperature ($\sim 2560^\circ\text{C}$) under 3.8-MPa tensile stress provided a reversible yarn contraction of 7.3% (Fig. 2B and movie S4), corresponding to 0.16 kJ/kg of contractile work capability per yarn weight. Because yarn coiling greatly enhanced actuation stroke, coiled yarns (Fig. 1E) are the focus of the studies on tensile actuation discussed below.

Tensile actuation at 1200 cycles per minute and 3% stroke was demonstrated for more than 1.4 million cycles (Fig. 3A) with a two-end-tethered, wax-filled, coiled Fermat yarn that lifted 17,700 times its own weight when powered by a 20-Hz, 18.3-V/cm square-wave voltage. Fast passive cooling in 25 ms resulted from the small yarn and coil diameters (11.5 and 20 μm , respectively). The performance of this yarn was optimized by increasing applied voltage and mechanical load, while reducing pulse duration. Figure 3B shows a series of actuations wherein the yarn lifts 175,000 times its mass in 30 ms when 32 V/cm was applied for 15 ms. The work during contraction (0.836 kJ/kg) provided a power output of 27.9 kW/kg, which is 85 times the peak output of mammalian skeletal muscles (0.323 kW/kg) (23) and 30 times the maximum measured power density of previous carbon nanotube muscles. However, the high applied electrical power reduces cycle life by causing excessive heating and paraffin evaporation.

Figure 3C shows the stress dependence of actuator stroke and work capacity for different amounts of twist insertion in a wax-infiltrated, 150- μm -diameter, dual-Archimedean yarn that is two-end tethered. Reversible contraction, which is greatly enhanced for yarn having sufficient twist to cause coiling, resulted from steady-state electrical heating to just below the wax vaporization temperature. Applying high stress decreases stroke, owing to the yarn's lower Young's modulus in the contracted state (containing molten wax)

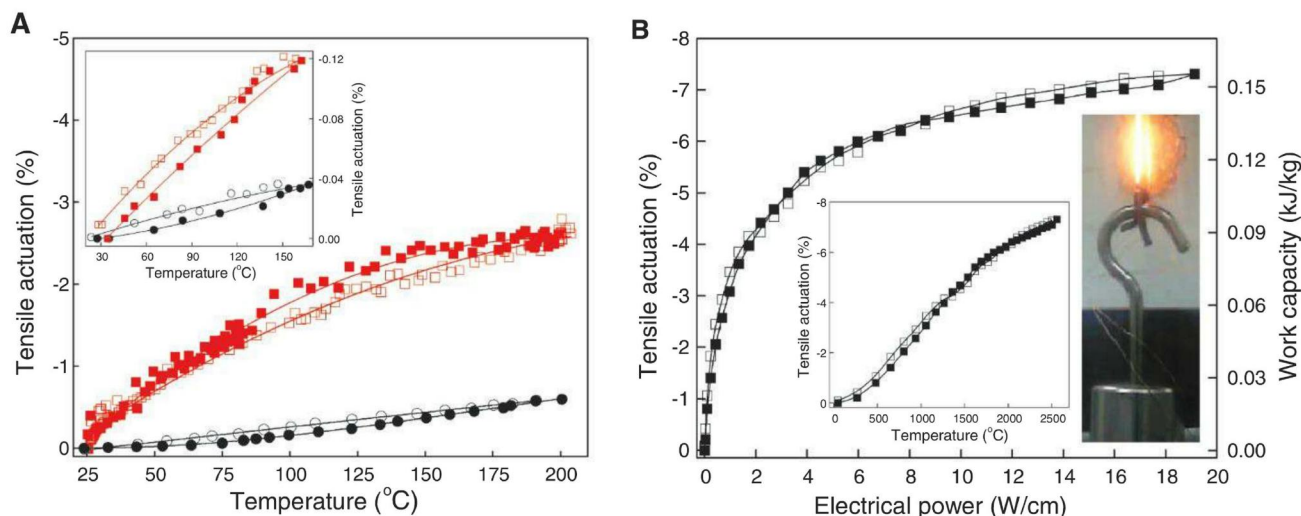


Fig. 2. Thermal tensile actuation for two-end-tethered homochiral yarns. (A) Tensile actuation strain versus temperature before (black) and after (red) wax infiltration for a coiled, dual-Archimedean yarn having 130- μm initial diameter, an inserted twist of ~ 4000 turns/m (per length of the precursor sheet stack), and an applied stress of 6.8 MPa. Inset: Corresponding actuation data before (black) and after (red) wax infiltration for a noncoiled Fermat yarn having 16- μm initial diameter, $\sim 20,000$ turns/m twist, and an applied stress of 4.8 MPa. (B) Electro-

thermal tensile actuation strain and work capacity during contraction in vacuum as a function of applied electrical power for a neat, coiled, dual-Archimedean yarn having 115- μm diameter and the inserted twist of the dual-Archimedean yarn in (A). Insets: Tensile actuation versus estimated temperature for this yarn (left) and photograph of the incandescent yarn lifting a 10-g load. Closed symbols and open symbols in (A) and (B) are for increasing and decreasing temperature, respectively.

and correspondingly larger elastic elongation under load than in the initial state (where the solid wax provides structural reinforcement for both tensile and torsional deformations) (fig. S2). The stroke for highly coiled yarn decreases at low stresses (Fig. 3C), which is consistent with the close proximity of adjacent coils hindering contraction.

Figure 3C shows that there is an optimal amount of coiling that maximizes either stroke or work during contraction for the wax hybrid yarn. A maximum contraction of 5.6% was observed at 5.7-MPa stress for a coiled Femat yarn having intermediate twist. Adding 6.8% more twist to the coiled yarn increased the stress of maximum contraction (16.4 MPa for 5.1% strain) and the maximum measured contractile work (1.36 kJ/kg at 84 MPa), which is 29 times the work capacity of natural muscle (24). Subsequently reducing twist by 41% eliminated coiling and reduced maximum contraction and contractile work to low values (0.7% and 0.31 kJ/kg, respectively). Contractions of 10% under 5.5-MPa stress were realized for a 150- μ m-diameter, partially coiled, dual-Archimedean yarn by applying well-separated 50-ms, 15-V/cm pulses (Fig. 3D). Because the cross-sectional area of this yarn was 170 times higher than for the yarn of Fig. 3, A and B, passive cooling in ambient air was less effective: The cooling time increased from 25 ms to ~2.5 s, resulting in a low contractile power density when both heating and cooling times are considered (0.12 kW/kg).

The highest presently realized ratio of the mechanical work done during contraction to the input electrical energy is 0.55% (25), which is similar to the energy conversion efficiency of commercially used shape memory metals, which can reach 1 or 2% (26). The energy efficiency for the hybrid yarn muscles can be increased by minimizing the thermal

energy loss during actuation, increasing the allowable mechanical load by increasing yarn strength, and increasing the ratio of guest volume change to the enthalpy change needed to produce it.

The Fig. 1, C and D, configurations provided highly reversible torsional and tensile actuation when the final actuation temperature (T_f) was below the temperature at which wax melting starts (T_{ms}). Actuation for these configurations became increasingly irreversible at higher temperatures for nonplied yarn, especially when mechanical load was large, and cycling to above T_{mf} (where melting is complete) caused large permanent untwist. This problem does not arise for SZ two-ply yarn (or its chiral opposite); because the sum of yarn Z twist and the S twist of yarn plying must be conserved, uncoiling during reversal of actuation acts as a torsional return spring for reversing twist release within the yarn (27) (fig. S9).

Very fast, highly reversible torsional actuation was demonstrated for 2 million cycles for a 6.9-cm-long, 10- μ m-diameter, two-end-tethered, half-wax-infiltrated homochiral Femat yarn that rotated a paddle at yarn midpoint (Fig. 1B configuration). The hybrid yarn accelerated a 16.5-times-heavier paddle to a full-cycle-averaged 11,500 rotations per minute—first in one direction and then in reverse (Fig. 4A). Even though actuation temperature was far above T_{mf} , this high cycle life resulted because of the presence of the unactuated yarn segment of Fig. 1B, whose twisting during the untwisting of the actuating yarn segment acted as a torsional return spring. Figure 4B shows the dependence of torsional rotation on input electrical power and applied tensile load for a similar yarn that rotated a 150-times-heavier paddle for a million highly re-

versible cycles. Increasing load increased rotation speed from 5500 revolutions/minute (movie S2) to a maximum of 7900 revolutions/minute. Reversible torsional actuation (12.6°/mm) was also driven for a half-wax-infiltrated yarn by replacing electrical heating with heating using light pulses (movie S4) from a 100-W incandescent lamp.

Torsional actuation of a fully infiltrated, heterochiral, noncoiled, dual-Archimedean yarn (Fig. 1D) was used to hurl a projectile by rotating the arm of a miniature Greco-Roman-style catapult by 300° (Fig. 4C and movie S3). A maximum specific torque of 8.42 N·m/kg was generated for this 100- μ m-diameter yarn, which is five times the value demonstrated for electrochemically driven nanotube yarn and slightly higher than for large electric motors (up to 6 N·m/kg). Though the maximum torsional actuation temperature was above T_{mf} and the yarn is heterochiral and fully infiltrated, reversible operation was achieved as the torsional rotation range was limited to 130° and the actuation temperature was below that of appreciable wax evaporation.

We have also demonstrated reversible, thermally powered torsional actuation for hybrid yarn containing other volume-expanding guests. One example is $\text{CH}_3(\text{CH}_2)_{11}\text{C}\equiv\text{C}-\text{C}\equiv\text{C}(\text{CH}_2)_8\text{COOH}$, which was infiltrated into twist-spun Femat yarn (diameter = 9 μ m and $\alpha = 30^\circ$) and photopolymerized by 1,4-addition. Similar to a related polydiacetylene used to make color-changing carbon nanotube yarns (28), the produced polydiacetylene is polychromatic, providing a reversible blue-to-red phase transition at ~57°C. Owing to a few percent volume increase at this blue-red phase transition and a larger volume change from melting incompletely polymerized monomer at 63°C,

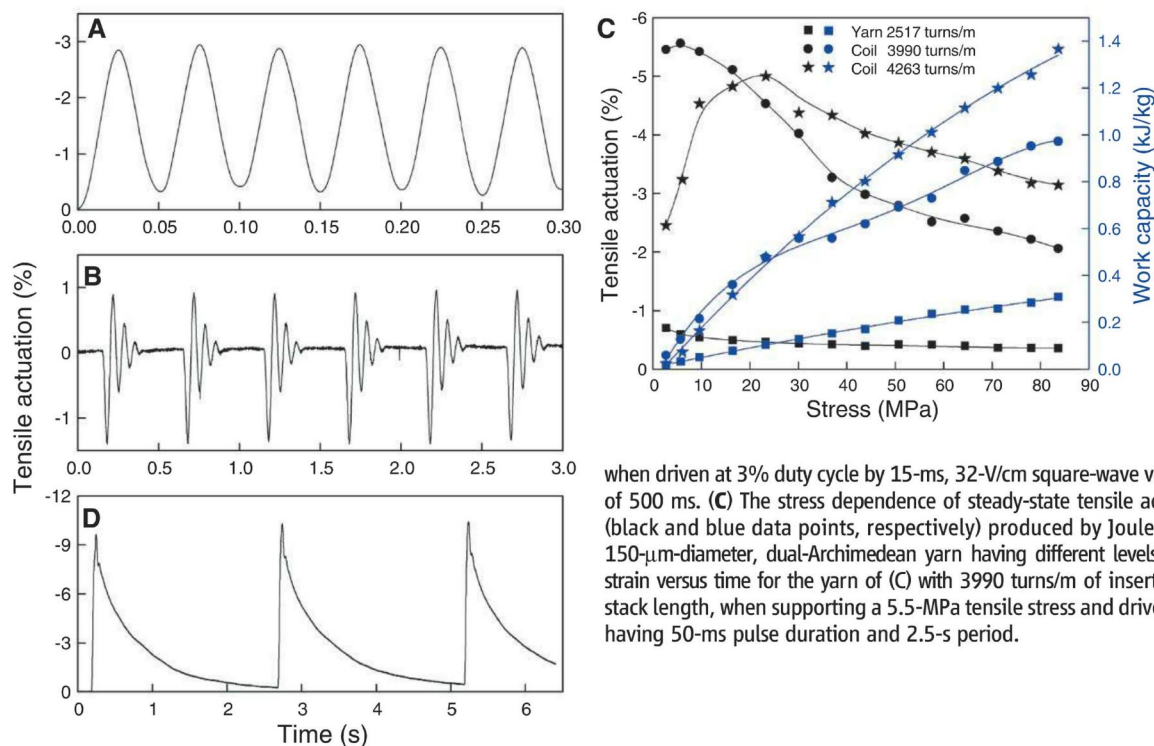


Fig. 3. Electrothermal tensile actuation for two-end-tethered, homochiral, wax-filled yarns. (A) Tensile actuation strain versus time after 1,400,000 reversible cycles for a 11.5- μ m-diameter, coiled Femat yarn having ~25,000 turns/m twist when driven by an 18.3-V/cm, 20-Hz symmetric square wave voltage while lifting a load that provided a 14.3-MPa stress. (B) Tensile actuation for the yarn of (A) with 109-MPa applied tensile stress

when driven at 3% duty cycle by 15-ms, 32-V/cm square-wave voltage pulses having a period of 500 ms. (C) The stress dependence of steady-state tensile actuation and contractile work (black and blue data points, respectively) produced by Joule heating (0.189 V/cm) for a 150- μ m-diameter, dual-Archimedean yarn having different levels of inserted twist. (D) Tensile strain versus time for the yarn of (C) with 3990 turns/m of inserted twist per precursor sheet stack length, when supporting a 5.5-MPa tensile stress and driven by a 15-V/cm square wave having 50-ms pulse duration and 2.5-s period.

reversible torsional rotation of $100^\circ/\text{mm}$ was obtained for actuation to below 80°C for the yarn configuration of Fig. 1B. Actuation to higher temperatures was poorly reversible, likely because of an irreversible phase transition.

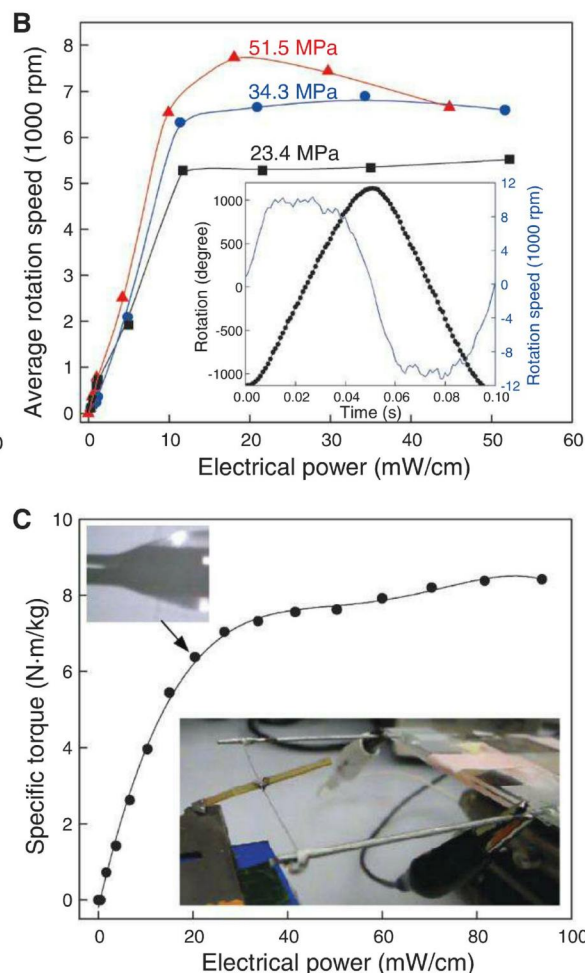
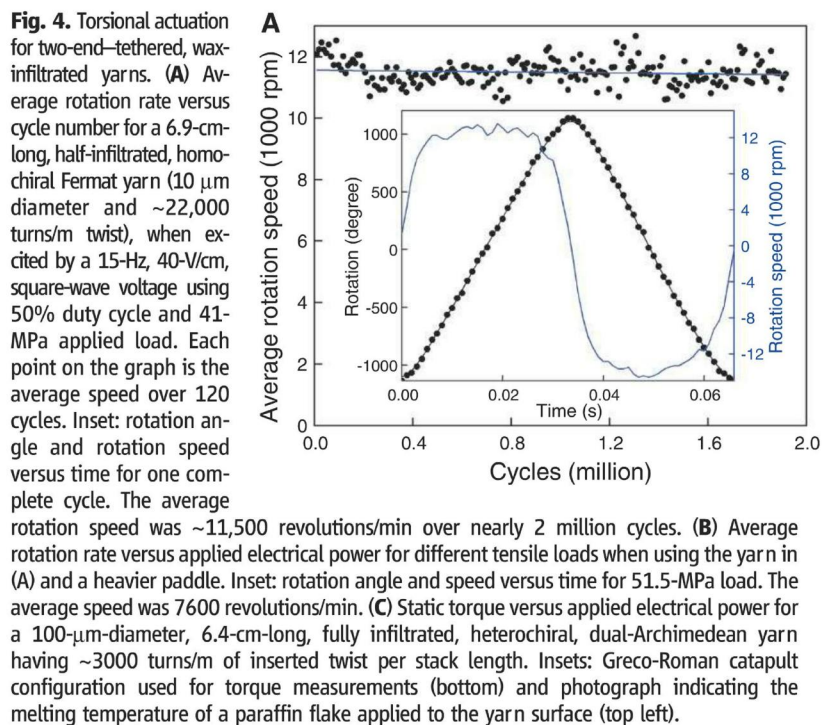
Actuation powered by absorption. Reversible torsional actuation was powered by the absorption and desorption of hydrogen on a 60-nm-thick palladium layer on nanotube bundles (fig. S4) within a dual-Archimedean yarn (16). Because this 144- μm -diameter yarn contained 90 weight (wt) % palladium, the resulting high torsional rigidity restricted twist insertion to ~ 200 turns/m. Nevertheless, a one-end-tethered yarn rotated at its free end a thousand-times-heavier paddle during hydrogen absorption. Injection of 0.05-atm H_2 into a vacuum chamber containing the actuator caused 1.5 paddle rotations within ~ 6 s, which was fully reversed on a similar time scale during repeated cycling between hydrogen exposure and vacuum. Cantilever-based actuators exploiting the dimensional changes of a 10- μm -thick Pd alloy layer have been previously demonstrated (29), but the response time was in tens of minutes. The yarn's 100-fold faster response rate resulted from yarn porosity and the thinness of the Pd coating. Such yarn actuators might be used as intelligent muscles that rapidly close an inlet when a targeted hydrogen pressure is exceeded.

Liquid absorption and desorption can also drive actuation, as shown in fig. S10, where torsional actuation of a two-end-tethered Fermat yarn is shown as a function of immersion length in liquid. As with a polymer that absorbs a liquid or vapor, the immersed yarn swells, and this volume change drives torsional actuation.

Discussion. The volume expansion of a liquid wax reversibly drives actuation rather than causing wax extrusion from the porous yarn because of the giant interfacial energies that arise on the nanoscale. The molten wax undergoes a fractional volume decrease $\Delta V_w/V_w$ when cooled. If this wax volume change occurred without decreasing yarn volume, nanotube-paraffin interfacial energies (γ_{np}) would be replaced by nanotube-air interfacial energies (γ_{na}) at an energy cost of $(\gamma_{na} - \gamma_{np})(\Delta V_w/V_w)A_n$, where A_n is the gravimetric surface area of the nanotubes. Using $\gamma_{na} - \gamma_{np} \sim 18 \text{ mJ/m}^2$ (30), $A_n \sim 97 \text{ m}^2/\text{g}$ (31), and $\Delta V_w/V_w \sim 0.2$, about 0.35 kJ/kg of energy is available to compress the nanotube yarn as the volume of the liquid wax decreases. During subsequent yarn actuation by heating and corresponding wax expansion, this elastic energy in the yarn is progressively released, thereby maintaining coincidence between molten wax and yarn volume over the entire actuation cycle—as is observed. This analysis correctly predicts that excess wax on the yarn surface, as well as wax evaporation, will decrease tensile stroke.

Why is tensile contraction no more than 0.7% for wax-filled, noncoiled, nonplied yarns undergoing volume expansions of about 10% (fig. S2), despite these same yarns providing high torsional actuation? Low tensile contraction results for a noncoiled, Fermat yarn because the yarn bias angle decreases toward zero with decreasing radius within the yarn (Eq. 1) and the fractional contraction ($\Delta L/L$) produced by a fractional volume change strongly depends upon bias angle (fig. S11). Length contraction of the outer yarn layer is dramatically reduced by dimensional mismatch with progressively smaller contractions closer to yarn center. This dimensional mismatch similarly limits tensile contraction for the component helical scrolls in dual-Archimedean yarn.

The thermal expansion coefficient for noncoiled, neat, Fermat yarn between 25° and 200°C (about $-2.2 \times 10^{-6}/^\circ\text{C}$) (Fig. 2A, inset) is similar to the in-plane thermal expansion coefficient of graphite (which has a minimum of $-1.4 \times 10^{-6}/^\circ\text{C}$ at about 3°C , increases to $-0.71 \times 10^{-6}/^\circ\text{C}$ at 200°C , and becomes positive above $\sim 380^\circ\text{C}$) (32). However, the tensile contraction of uncoiled neat yarn becomes 2.8 times as negative with increasing twist (fig. S1), indicating that contraction of nanotube length cannot fully explain yarn contraction. This yarn contraction is relatively insensitive to mechanical load and is approximately the same for



neat Fermat and dual-Archimedean yarns having about the same diameter and bias angle (fig. S2).

Diverse structural effects can potentially contribute to tensile actuation for coiled, two-end-tethered, nonplied yarns, including conversion between twist and writhe (in both uncoiled and coiled regions) and changes in coil diameter, pitch, and yarn length. Although twist-to-writhe conversion (corresponding to an increase in number of coils) would enhance thermal contraction during actuation, optical microscopy indicates that total coil number does not measurably increase during actuation for either heavily or lightly coiled, wax-filled, dual-Archimedean yarns. These results suggest that tensile contraction is predominantly caused by a decrease in separation between neighboring coils.

Yarn coiling increases the negative thermal expansion of a neat twist-spun yarn by a factor of ~10. Because these coiled neat yarns provide up to 7.3% hysteresis-free contraction when lifting heavy loads using temperature changes up to ~2560°C (Fig. 2B and movie S4), these muscles can be deployed in inert atmosphere to temperatures at which no other high-work-capacity actuator can survive.

For applications in which yarn-size torsional and tensile actuators are needed, the absence of electrolyte and associated packaging, the low required voltages, and the high cycle life and energy and power densities suggest the possibility of early commercial deployment. The main competing technology of NiTi shape memory metal actuators provides highly hysteretic actuator strokes; actuator control is complicated by the dependence of stroke on prior history within a cycle (26). This history dependence is small for the wax hybrid yarn results of Fig. 2A and should be negligible for cycling a neat yarn or any wax-filled yarn between molten states. However, as with shape memory metal wires and other thermally powered actuators (26), electrothermal energy conversion efficiency is low. Future pos-

sibilities include environmentally powered hybrid muscles that open textile pores or close window blinds when it is too hot, or actuate in response to agents in the environment.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/338/6109/928/DC1
Supplementary Text
Figs. S1 to S11
References (33–37)
Movies S1 to S5

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REPORTS

Synthetic Lipid Membrane Channels Formed by Designed DNA Nanostructures

Martin Langecker,^{1*} Vera Arnaut,^{1*} Thomas G. Martin,^{2*} Jonathan List,¹ Stephan Renner,¹ Michael Mayer,³ Hendrik Dietz,^{2†} Friedrich C. Simmel^{1†}

We created nanometer-scale transmembrane channels in lipid bilayers by means of self-assembled DNA-based nanostructures. Scaffolded DNA origami was used to create a stem that penetrated and spanned a lipid membrane, as well as a barrel-shaped cap that adhered to the membrane, in part via 26 cholesterol moieties. In single-channel electrophysiological measurements, we found similarities to the response of natural ion channels, such as conductances on the order of 1 nanosiemens and channel gating. More pronounced gating was seen for mutations in which a single DNA strand of the stem protruded into the channel. Single-molecule translocation experiments show that the synthetic channels can be used to discriminate single DNA molecules.

A large class of proteins and peptides form channels through lipid bilayer membranes (1) to facilitate the transport of water, ions,

or other entities through the otherwise impermeable membranes. Here, we report on a synthetic membrane channel that is constructed entirely

from DNA and anchored to a lipid membrane by cholesterol side chains. The shape of our synthetic channel is inspired by the natural channel protein α -hemolysin (2), although there are differences in physical properties such as charge, hydrophobicity, and size.

We constructed the channel by means of molecular self-assembly with scaffolded DNA origami (3–9) (Fig. 1A). The channel consists of two modules: (i) a stem that penetrates and spans a lipid membrane, and (ii) a barrel-shaped cap

¹Lehrstuhl für Bioelektronik, Physics Department and ZNN/WSI, Technische Universität München, 85748 Garching, Germany.

²Walter Schottky Institute, Physics Department, Technische Universität München, 85748 Garching, Germany. ³Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109, USA.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: dietz@tum.de (H.D.); simmel@tum.de (F.C.S.)

that adheres to the cis side of the membrane. Adhesion to the lipid bilayer is mediated by 26 cholesterol moieties that are attached to the cis-facing surface of the barrel (Fig. 1A). The stem protrudes centrally from the barrel and consists of six double-helical DNA domains that form a hollow tube. The interior of this tube acts as a transmembrane channel, with a diameter of ~ 2 nm and a length of ~ 42 nm, that runs through both stem and barrel (Fig. 1, B and C).

Transmission electron microscopy (TEM) images taken from purified structures (Fig. 1D) confirmed that the intended shape is realized (text S1

and figs. S1 and S2) (10). Experiments with unilamellar lipid vesicles show that the synthetic DNA channels bind to lipid bilayer membranes (Fig. 1, E to G, text S2, and figs. S4 to S8) in the desired orientation in which the cholesterol-modified face of the barrel forms a tight contact with the membrane and the stem appears to protrude into the lipid bilayer (Fig. 1F and fig. S9).

These observations suggested that the synthetic DNA channels could form membrane pores as designed. Because the energetic cost for insertion of the charged DNA structure into the

hydrophobic core of the lipid membrane would be prohibitively high, membrane penetration is thought to involve reorganization of the lipid bilayer around the charged stem structure, with the hydrophilic lipid head groups oriented toward the DNA structure (see text S3).

To demonstrate the electrical conductivity of the resulting membrane pores, we performed single-channel electrophysiological experiments (11) using an integrated chip-based setup (fig. S14) (12). We added synthetic DNA channels at low concentrations (~ 200 pM) to the cis side of the setup and applied voltage pulses to facilitate

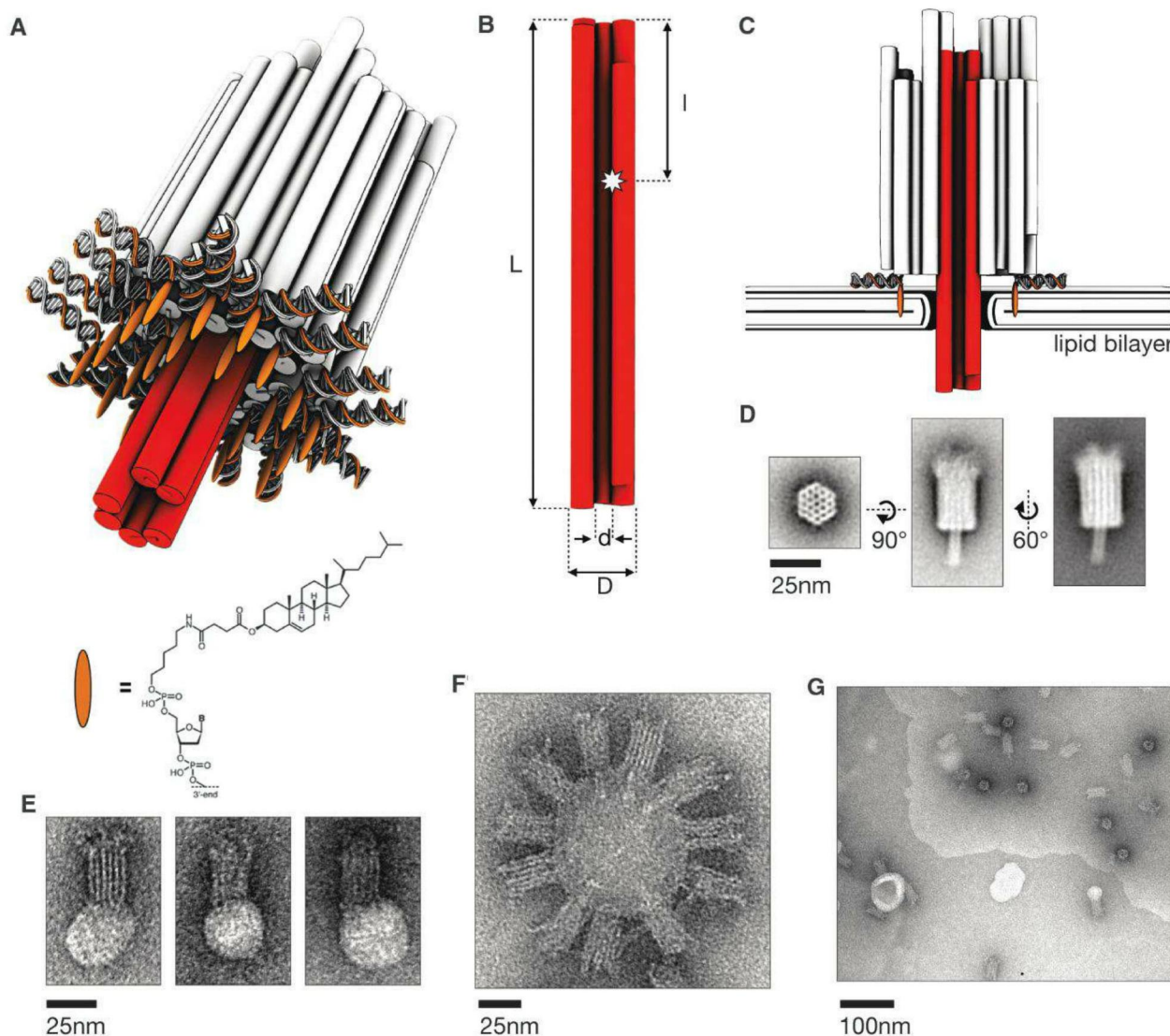


Fig. 1. Synthetic DNA membrane channels. (A) Schematic illustration of the channel formed by 54 double-helical DNA domains packed on a honeycomb lattice. Cylinders indicate double-helical DNA domains. Red denotes transmembrane stem; orange strands with orange ellipsoids indicate cholesterol-modified oligonucleotides that hybridize to single-stranded DNA adaptor strands. (B) Geometric specifications of the transmembrane channel. Length $L = 47$ nm, tube diameter $D = 6$ nm, inner diameter $d = 2$ nm. The length of the central channel fully surrounded by DNA helices is 42 nm. The star symbol indicates the position of a 7-base strand extension acting as a "defect" in channel "mutants" [$l = 15$ nm for mutant M1 and $l = 14$ nm for mutant M2; see text S6 and caDNA maps

(figs. S22 to S24)]. (C) Cross-sectional view through the channel when incorporated in a lipid bilayer. (D) Averaged negative-stain TEM images obtained from purified DNA channel structures (class averages obtained from raw images displayed in figs. S1 and S2). (E and F) Example TEM images of DNA channels adhering to small unilamellar vesicles (SUVs) made from POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) lipids. More images and a statistical analysis of vesicle size distribution and binding efficiency are found in text S2 and figs. S4 to S9. (G) TEM image of DNA channels binding to an extended lipid bilayer in the upper right part of the image. DNA channels are found predominantly on lipid-covered areas or sticking to SUVs (see text S2).

incorporation into the membrane (13, 14) (fig. S11A). As with biological channels, successful membrane incorporation of individual synthetic DNA channels manifested itself in a stepwise increase in transmembrane current (Fig. 2A) along with an increase in electrical noise (fig. S11B). Depending on the preparation method, we also observed incorporation of multiple DNA channels into the same membrane (fig. S13). The synthetic DNA channels displayed an average ohmic conductance of $G = 0.87 \pm 0.15$ nS (ionic current $I = 174$ pA at 200 mV) per channel in a solution containing 1 M KCl and 2 mM MgCl_2 (Fig. 2, B and C). A simple geometrical model (1) predicts $G = 0.78$ nS for a channel with diameter of 2 nm and length of 42 nm (text S4), which agrees favorably with what we observe.

Many natural ion channels display current gating caused by switching between distinct channel conformations with different conductances (1). The synthetic DNA channels also displayed gating behavior (Fig. 2D, traces i to iii), which may be caused by thermal fluctuations of the structure. We hypothesized that stochastic unzipping and reziping of short double-helical DNA domains in the channel may also contribute to the observed current gating. To test this idea, we designed three channel “mutants” that differed from the “wild-type” channel only by a single-stranded heptanucleotide protruding from the central transmembrane tube (Fig. 1B and text S6). The mutant channels showed more pronounced gating than did wild-type channels (Fig. 2D, traces iv to vi), and they also significantly differed in their gating time statistics (Fig. 2E and fig. S16). Every investigated mutant channel displayed gating, whereas some of the wild-type channels did not show gating at all (Fig. 2D, trace i). Hence, the transmembrane current depended on fine structural details of the synthetic DNA channel.

In the past, nanoscale membrane pores have shown great potential for use as single-molecule biosensors (15–22), whose operation principle is based on the transient blockage of ionic current by analyte molecules. The type of sensing task that can be accomplished with such “nanopores” depends on their size and their chemical structure. Nanopore sensors based on naturally occurring membrane pores provide excellent electrical properties, but altering the geometry of biological pores and introducing chemical functions through genetic engineering or chemical conjugation is challenging. By contrast, the geometry of synthetic DNA objects (23, 24) and their chemical properties (24) can be tailored for custom nanopore sensing applications. Here, we used our synthetic DNA lipid membrane channel for single-molecule studies of DNA hairpin unzipping and guanine quadruplex (25) unfolding. Single-stranded DNA is expected to fit through the 2-nm central pore of the DNA channel, whereas larger DNA secondary structures such as hairpins or quadruplexes are not (26, 27). To translocate through the DNA channel, the structures must unzip or unfold as in

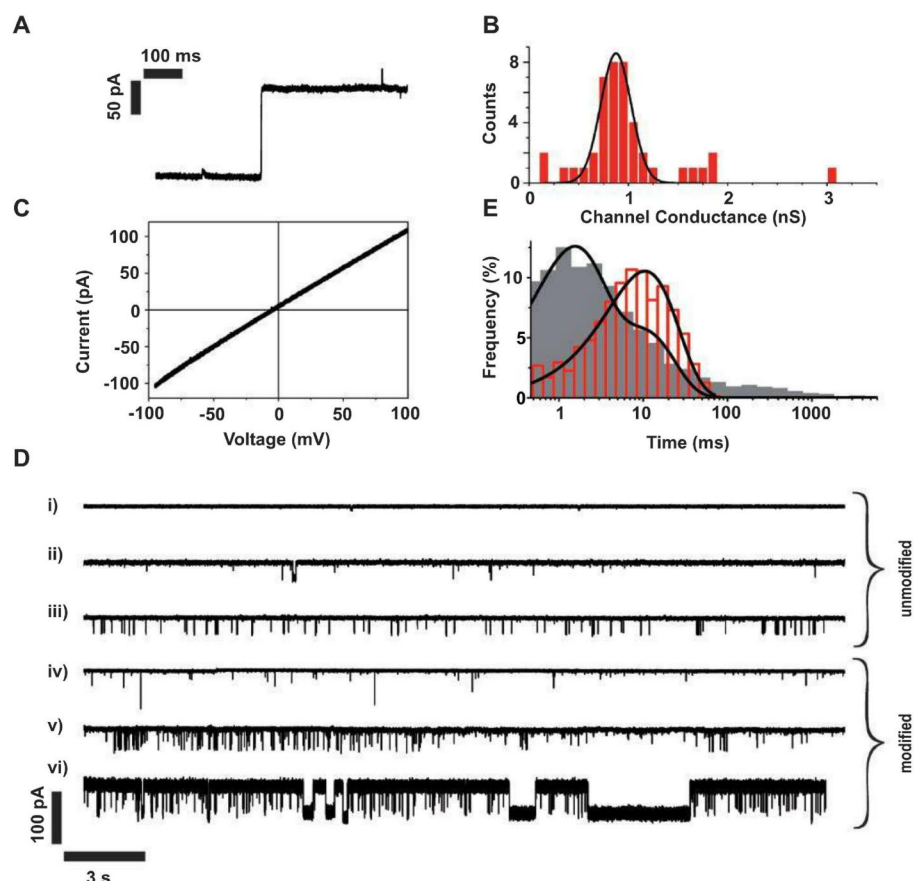


Fig. 2. Electrical characterization performed on painted DPhPC (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine) bilayers on a chip-based electrophysiology setup (an Ionera MECA-16 chip on a Nanion Orbit platform). **(A)** Stepwise increase in ionic current during an incorporation event at $V = 200$ mV. **(B)** Histogram of channel conductances obtained from 43 incorporation events. The black line depicts a Gaussian fit. **(C)** Current-voltage dependence of the channel after incorporation. **(D)** Typical current traces obtained from “wild-type” channels (i to iii) and “mutants” with modification M2 (iv to vi) (see text S6). **(E)** Statistics of gating times for the wild-type (red) and mutant (gray) channels. Continuous lines correspond to a monoexponential fit for the wild-type channel ($\tau = 10.5$ ms) and a double-exponential fit for the mutant ($\tau_1 = 1.3$ ms, $\tau_2 = 9.2$ ms).

similar experiments with α -hemolysin, thus providing a characteristic time delay in the current blockades that reveals the kinetics of structural transitions (26–28).

For one set of experiments, we used a DNA hairpin with a 9-base pair stem flanked by 50 thymidines on the 3' end and 6 thymidines on the 5' end (Fig. 3A). The hairpin molecules were initially added to the cis side of a lipid membrane containing a single synthetic DNA channel that displayed a stable current baseline without gating. Application of a positive voltage bias led to capture, unzipping, and translocation of the hairpin structures, resulting in transient current blockades (Fig. 3, A and B). Reversal of the bias after ~30 min again led to transient current blockades, this time caused by molecules that had accumulated in the trans compartment by previous translocation through the DNA channel. The blockade amplitudes for both translocation directions were $\Delta I_{\text{cis-trans}} = 11.9 \pm 2.7$ pA and $\Delta I_{\text{trans-cis}} = 20.3 \pm 4.2$ pA. The blockade dwell times were distributed exponentially, with a characteristic life-

time of $\tau_{\text{cis-trans}} = 1.5$ ms and $\tau_{\text{trans-cis}} = 1$ ms, respectively (Fig. 3C).

In another set of experiments, we added quadruplex-forming oligonucleotides with a single-stranded tail consisting of 60 thymidines (Q-T60) to the cis side of a membrane containing a single synthetic DNA channel (Fig. 3, D and E). Again we observed transient current blockades, which correspond to the capture and threading of quadruplex DNA molecules into the channel, followed by unfolding and subsequent translocation through the pore. Removal of the analyte from the cis compartment restored a stable baseline current without blockades. Subsequent addition of quadruplex DNA with a longer 125-deoxythymidine (dT) tail (Q-T125) led to larger current blockades. The average current blockades were $\Delta I_{\text{Q-T60}} = 5.6 \pm 1.0$ pA and $\Delta I_{\text{Q-T125}} = 15.3 \pm 2.3$ pA, respectively. The larger current blockade for Q-T125 relative to Q-T60 translocations can be explained by the larger volume occupied in the channel by the longer T₁₂₅ tail than by the T₆₀ tail (text S7). The blockade dwell times were distributed

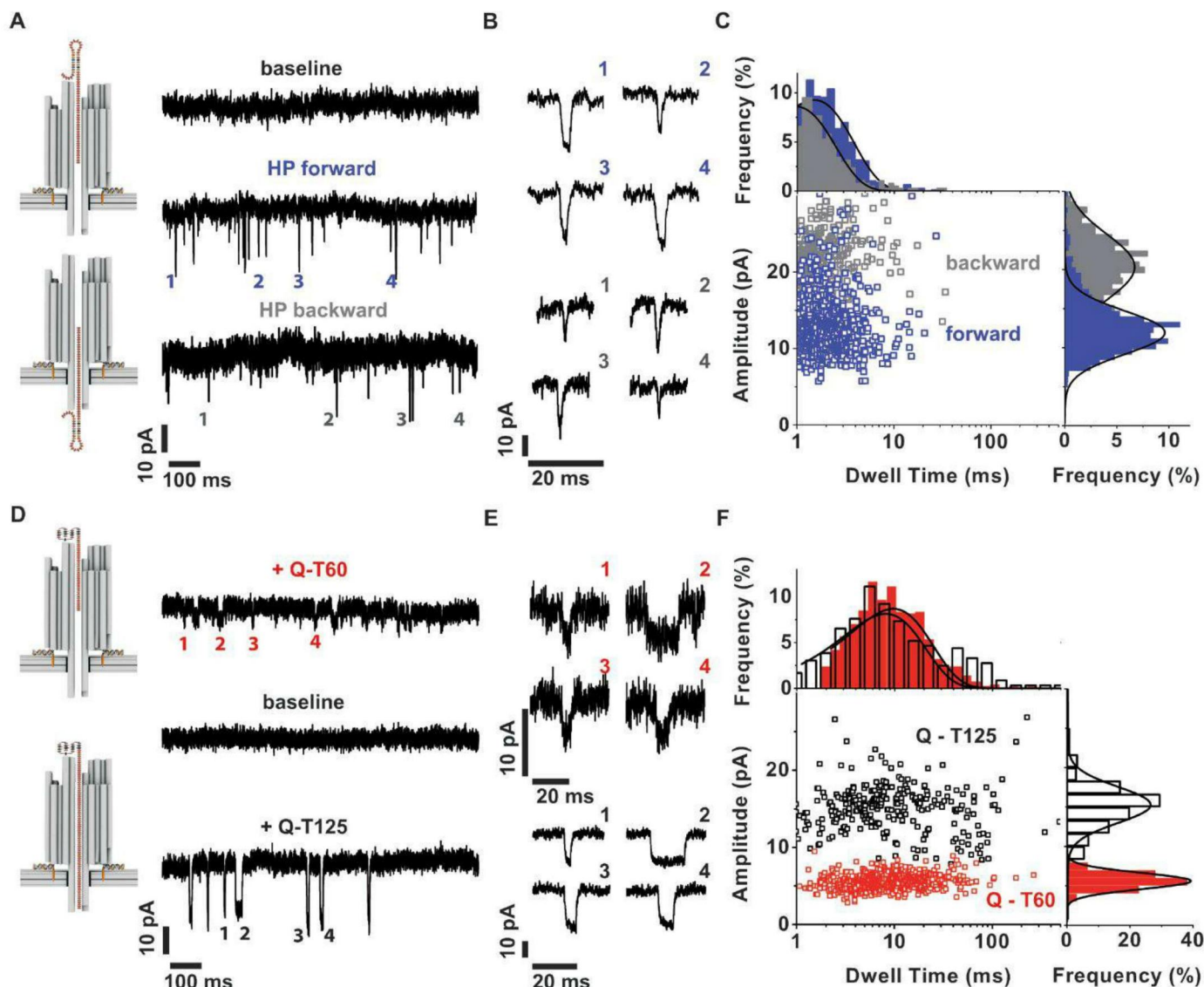


Fig. 3. DNA translocation studies. (A) Addition of DNA hairpins (T5-HP-T50) to a DNA channel at $V = 200$ mV results in the appearance of current blockades, indicating unzipping and translocation of hairpin molecules from cis to trans. Hairpins accumulated on the trans side can also be transferred back from trans to cis by a reversal of the transmembrane voltage. (B) Representative blockade events for forward (top) and backward (bottom) translocation of DNA hairpins. (C) Scatterplot for the translocation of T5-HP-T50 DNA through a DNA channel from cis to trans (blue) and from trans to cis side (gray) at $V = 200$ mV, and corresponding histograms. Each data point corresponds to a single translocation event. In total, 777 forward and 379

backward events were analyzed. (D) Top: Typical current trace at $V = 200$ mV after addition of $10 \mu\text{M}$ Q-T60 DNA. Middle: Current trace after rinsing with buffer solution. Bottom: Current trace after subsequent addition of $10 \mu\text{M}$ Q-T125 DNA to the same channel. (E) Representative blockade events for Q-T60 DNA (top) and Q-T125 DNA (bottom). (F) Scatterplot of current blockade versus dwell time for the translocation of Q-T60 DNA (red) and Q-T125 DNA (black) through the DNA channel. In total, 631 Q-T60 events and 279 Q-T125 events were analyzed; corresponding histograms are shown; the lines correspond to single-exponential fits (top histogram) and Gaussian fits (right histograms).

exponentially, with characteristic lifetimes of $\tau_{\text{Q-T60}} = 9.7$ ms and $\tau_{\text{Q-T125}} = 8.1$ ms. Thus, like biological pores, our synthetic DNA channels can be used as sensing devices to discriminate analyte molecules by studying their translocation characteristics.

In addition to single-molecule sensing, the synthetic DNA channels introduced here open up broad perspectives for applications as antimicrobial agents and interference with cellular homeostasis. More generally, we believe that fully synthetic lipid membrane channels are a crucial first step toward harnessing ion flux for

driving sophisticated nanodevices inspired by the rich functional diversity of natural membrane machines, such as ion pumps, rotary motors, and transport proteins.

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electrophysiological experiments; T.G.M. prepared the synthetic DNA channels and performed TEM; J.L., V.A., and T.G.M. performed experiments with lipid vesicles; and H.D. and F.C.S. wrote the paper. All authors discussed the results and commented on the manuscript.

Supplementary Materials

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Materials and Methods
Texts S1 to S9
Figs. S1 to S24
Tables S1 to S3
References (29–46)

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Coherent Phonon Heat Conduction in Superlattices

Maria N. Luckyanova,^{1*} Jivtesh Garg,^{1*} Keivan Esfarjani,¹ Adam Jandl,² Mayank T. Bulsara,² Aaron J. Schmidt,³ Austin J. Minnich,⁴ Shuo Chen,⁵ Mildred S. Dresselhaus,^{6,7} Zhifeng Ren,⁵ Eugene A. Fitzgerald,² Gang Chen^{1†}

The control of heat conduction through the manipulation of phonons as coherent waves in solids is of fundamental interest and could also be exploited in applications, but coherent heat conduction has not been experimentally confirmed. We report the experimental observation of coherent heat conduction through the use of finite-thickness superlattices with varying numbers of periods. The measured thermal conductivity increased linearly with increasing total superlattice thickness over a temperature range from 30 to 150 kelvin, which is consistent with a coherent phonon heat conduction process. First-principles and Green's function–based simulations further support this coherent transport model. Accessing the coherent heat conduction regime opens a new venue for phonon engineering for an array of applications.

Heat conduction usually occurs by a random walk of thermal energy carriers such as phonons, electrons, or molecules. During the last two decades, size effects on phonon heat conduction that lead to a deviation from this random walk behavior have drawn considerable attention (1). Most experimental observations of phonon size effects can be explained by invoking the Casimir picture, wherein phonons travel ballistically or quasi-ballistically through the internal region of the specimen and scatter at interfaces and boundaries (2). Such classical size effects are important for a wide range of applications including

thermoelectric energy conversion and micro-electronic thermal management.

In this classical size regime, the phase information carried by phonons is lost through the diffuse scattering of phonons at boundaries and by internal scattering processes. However, it should be possible to control the conduction of heat by manipulating phonon waves through, for example, stop-band formation in periodic structures (3), soliton waves (4), and phonon localization (5). Such manipulations require that heat-carrying phonons maintain their phase information throughout the heat conduction process. Coherent phonons in superlattices (SLs) have been observed with

Raman and acoustic reflection and transmission experiments, but these experiments probe a single frequency (6–8), rather than the integrated distribution associated with heat transfer. Thus, a conclusive demonstration of coherent phonon heat conduction remains an open challenge.

The term “coherent” has different meanings in different fields. Typically, it is used to characterize the source of a nearly monochromatic wave and implies a measurable phase relationship for a given time interval during wave propagation. Although this definition applies to a monochromatic wave, it does not apply to heat conduction, which involves all the thermally excited phonons in a structure. To clarify the meaning of coherent heat conduction, we consider heat conduction across the thickness of a thin film. In the Casimir classical size effect regime, broadband phonons thermally excited at one boundary traverse the

¹Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. ²Department of Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. ³Department of Mechanical Engineering, Boston University, Boston, MA 02215, USA. ⁴Division of Engineering and Applied Science, California Institute of Technology, Pasadena, CA 91125, USA. ⁵Department of Physics, Boston College, Chestnut Hill, MA 02467, USA. ⁶Department of Physics, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. ⁷Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: gchen2@mit.edu

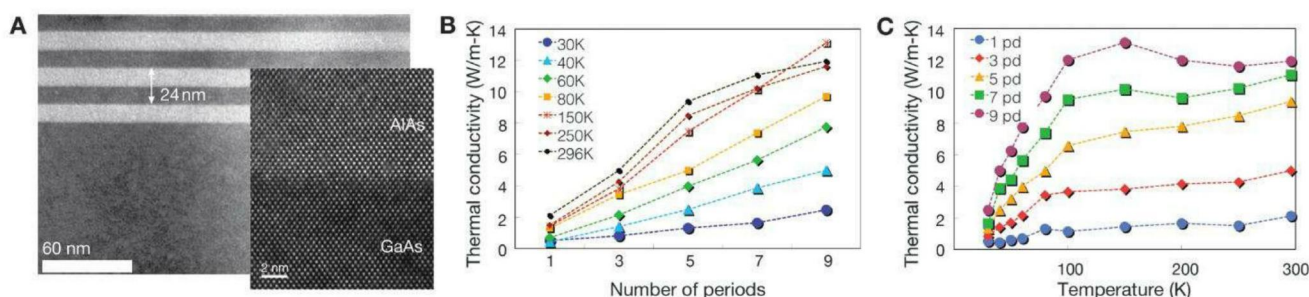


Fig. 1. (A) Cross-sectional TEM image of the 3-period (pd) SL. (Inset) HRTEM image of one of the interfaces. Measured thermal conductivity of GaAs/AlAs SLs as a function of (B) number of periods in the SL for different temperatures and (C) temperature for different SL thicknesses. If the interfaces in the SLs

destroy the phonon coherence, the measured thermal conductivity is expected to be independent of the number of periods. Below 150 K, the linearity of the thermal conductivity versus length suggests that phonon heat conduction in these SLs is coherent.

internal region of the film ballistically. Upon reaching the sample boundary, they are usually scattered diffusely. Hence, phonon propagation inside the layer is coherent. However, because boundaries often scatter phonons diffusely, it is commonly assumed that such scattering randomizes the phases of phonons such that interference effects, and the resultant modification of the phonon dispersion, can be neglected. For a SL with many internal interfaces, the key question for heat conduction is whether each internal interface behaves as a diffuse boundary, as in the classical size effect regime, or whether it behaves as a new material with its own phonon dispersion caused by interference of phonon waves propa-

gating through the whole structure. In the former case, phonon transport is incoherent and the SL can be thought of as a composite. In the latter case, phonon transport is coherent and the SL should be treated as one homogeneous material.

The idea of using periodic structures such as SLs and phononic crystals to control thermal transport by manipulating coherent phonons has existed for some time (9). Past experimental and theoretical studies, particularly those of SLs, have made such control seem unattainable except at very low temperatures (9, 10). For example, in SLs, extensive experimental and theoretical studies, which have focused on changing the periodicity, have shown that interface roughness and the re-

sulting diffuse scattering play a dominant role in the experimentally observed thermal conductivity reduction compared with their bulk parent materials (11–17), implying that phonon transport through SLs is mainly incoherent. We now show that coherent heat conduction occurs in SLs, despite the dominance of diffuse interface scattering in reducing the phonon thermal conductivity. The issue of coherent versus incoherent transport is important for understanding energy transport in a variety of disciplines.

We have taken a different experimental approach to study heat transport through SLs. Rather than changing the thickness of the SL periods, we measure the thermal transport properties of SLs with a constant period but a varying number of periods. If each internal interface scatters phonons diffusely, thus destroying their phase information, then the interface behaves like a thermal resistor because of the Kapitza resistance (18), such that many equivalent interfaces in series lead to an effective SL thermal conductivity, in the direction perpendicular to the interfaces, that is approximately independent of the number of layers (15). However, if the phase of the phonon is preserved at the interfaces of the SL and if anharmonic scattering is minimal, superposition of the Bloch waves creates stop bands and effectively modifies the phonon band structure (10).

In this regime, phonon mean free paths (MFPs) are equal to the sample length, leading to a thermal conductivity that is linearly proportional to the total thickness of the SL. This SL structure has the equivalent of a constant conductance through all the layers, as would be expected in a thin film with no internal scattering (19). In the case of a single material system, this regime is typically referred to as the “ballistic” transport regime. In the case of SLs, this implies that these phonons are eigenmodes of the SL and therefore have the modified dispersion arising from coherent wave interference effects. Therefore, in the case of SLs, ballistic transport across the whole thickness means coherent heat conduction in the SL. We should caution not to equate ballistic heat conduction to coherent heat conduction in general, because ballistic heat conduction is widely used to describe the reduced thermal conductivity of nanowires caused by diffuse scattering of phonons at the boundaries (20).

We used metal-organic chemical vapor deposition (MOCVD) to fabricate five GaAs/AlAs SL samples consisting of 1, 3, 5, 7, and 9 periods, where each period consists of 12 nm of GaAs and 12 nm of AlAs. Cross-sectional transmission electron microscope (TEM) images of the samples, such as Fig. 1A, were used to confirm the thickness of the layers, and high-resolution (HR) TEM images such as the inset in Fig. 1A confirmed the good quality of the interfaces between the layers. HRTEM images have low contrast for dilute concentrations of GaAs in AlAs, and vice versa, which makes determining the exact amount of interface mixing somewhat difficult. Recent

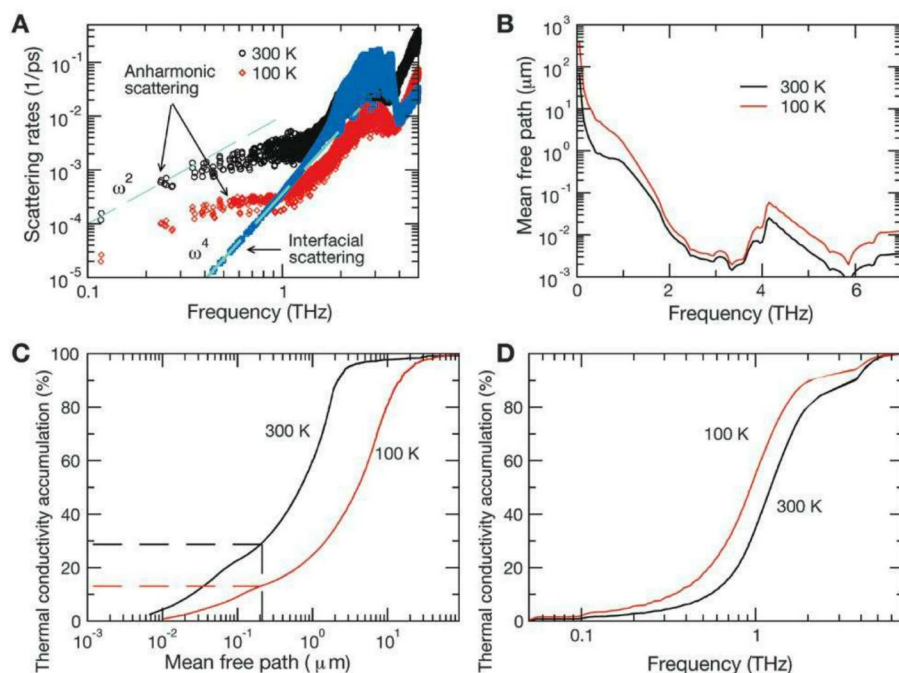


Fig. 2. Calculated first-principles results for an infinite 12 nm by 12 nm GaAs/AlAs SL. **(A)** Comparison between anharmonic (red for 100 K and black for 300 K) and interface (blue) scattering rates. Dashed blue lines are the fits to the scattering rates describing the ω^2 and ω^4 behaviors of the anharmonic and interfacial scattering, respectively, in the low-frequency regime. **(B)** Phonon MFPs in the SL as a function of frequency. Thermal conductivity accumulation in the SL as a function of **(C)** phonon MFP and **(D)** frequency, at 100 and 300 K.

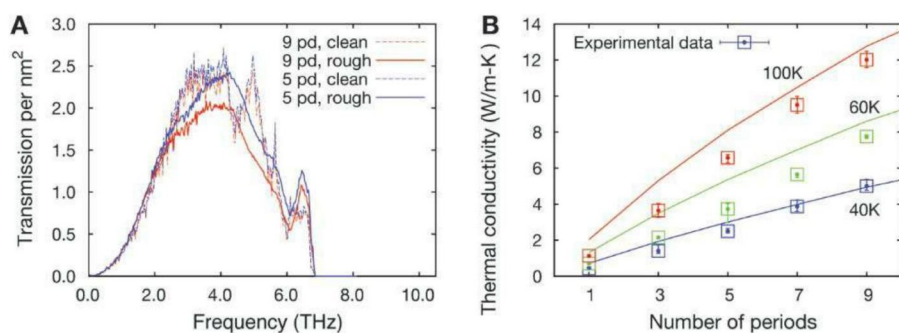


Fig. 3. **(A)** Transmission function per nm² of the 5- and 9-period SLs in the clean and rough boundary cases. **(B)** Thermal conductivity as a function of length at 40, 60, and 100 K for an SL with rough interfaces (solid) and experimental samples (dots with error bars).

scanning TEM (STEM) studies of GaAs/AlAs SLs grown by molecular beam epitaxy—have found interface mixing to extend over three atomic layers (21). The samples used in this study are expected to have slightly greater mixing due to the higher temperatures used in MOCVD.

We used time-domain thermoreflectance (TDTR) measurements to find the thermal conductivities of these samples (13, 22) at temperatures between 30 and 300 K. The experiment was performed between 30 and 300 K with liquid He cooling. The TDTR experimental setup used herein was discussed in greater detail in the relevant references (23–25).

The thermal conductivities of the SL samples resulting from the TDTR experiment are given in Fig. 1, B and C. The conductivity is linear in total SL thickness from 30 to 150 K (Fig. 1B). This nearly linear dependence of thermal conductivity on number of SL periods implies coherent phonon transport through the layers, whereas the nonlinear dependence at temperatures greater than 150 K suggests the increasing influence of incoherent effects. However, because the thermal conductivity is still increasing with the period number even at 296 K, coherent phonons still conduct a considerable fraction of heat at this temperature. The effective thermal conductivities of these SLs are much smaller than that of their bulk counterparts [the room temperature thermal conductivity of GaAs is 45 W/m-K and that of AlAs is 90 W/m-K (26)] and are increasing with temperature.

To understand the experimental results, we used a first-principles approach based on lattice dynamics to calculate the phonon MFPs of an infinite SL at different temperatures. Unlike past theoretical approaches based on the Boltzmann transport equation (15), lattice dynamics (27, 28), molecular dynamics (16, 29, 30), and acoustic wave equations that relied on various assumptions and fitting parameters, our first-principles approach relies on the use of harmonic and anharmonic interatomic force constants derived from density-functional perturbation theory (DFPT) (31) along with a solution of the Boltzmann transport equation to predict the thermal conductivity. Its use has been shown to lead to excellent agreement between experiments and theory for perfect crystals (32, 33) as well as alloys (34). More recently, Garg *et al.* (35) used this approach to compute the thermal conductivity of ideal Si/Ge SLs with perfect interfaces where phonons were taken to scatter only through anharmonic processes. The computed thermal conductivity values were higher than experimental values, indicating the need to incorporate the effects of interfacial disorder.

In this work, we include both interface roughness as the random mixing of Ga and Al atoms in a narrow region around the interface and three-phonon processes from first-principles without introducing any fitting parameters. Scattering caused by this mixing was computed from Fermi's golden rule (36).

Based on the results of these first-principles computations, we can extract detailed information such as the phonon MFP distribution and the MFP dependence of the thermal conductivity. Although these calculations were performed for an infinite SL, the information obtained regarding phonon MFPs allowed us to gain insight into the role of coherent transport in finite-size SLs. Figure 2A shows the anharmonic and interface scattering rates at different frequencies. Although lower-frequency phonons were mostly scattered through three-phonon processes, higher-frequency phonons were also scattered by the interfacial disorder. This interfacial scattering of high-frequency phonons led to a reduction in their heat-carrying ability and caused an overall decrease in the thermal conductivity of SLs. Low-frequency phonons had long MFPs (Fig. 2B) and could propagate through the entire SL structure and thus conduct heat coherently through a SL whose thickness was shorter than the MFPs. The contribution of these long MFP phonons to the total thermal conductivity is shown in Fig. 2, C and D, where thermal conductivity accumulation is plotted against MFP and frequency, respectively. Phonons with MFPs longer than 216 nm, the thickest measured SL, contributed 87% (at 100 K) and 71% (at 300 K) to the total thermal conductivity of an infinite SL, as indicated by the dashed lines in Fig. 2C. This finding points to the important role that low-frequency, long-MFP phonons play in heat transport through the SLs. These phonons form a new band structure with altered group velocities (fig. S3) and stop bands.

Further evidence of the dominant role of coherent phonon transport in finite SLs is provided by a Green's function (GF)-based calculation of the SL thermal conductivities (37–39). This theory is purely harmonic but rigorously includes roughness effects and the finite thickness of the SLs. Because first-principles results have shown that most of the heat is conducted by low-frequency phonons that are only weakly scattered by anharmonic processes in the bulk, we expect the GF treatment, which ignores this anharmonicity, to be valid (27). The experimental results indicate that for the finite-thickness SLs, phonon transport was mostly coherent. In this regime, similar to the Casimir regime, the thermal conductivity of the SL was mainly determined by scattering at the sample boundaries rather than at internal interfaces.

In Fig. 3A, we show the phonon transmission function across a 5- and 9-period SL sandwiched between semi-infinite Al and GaAs leads where both clean and rough SL interfaces are considered. Low-frequency phonons were not affected by interface roughness because their wavelengths were longer than the scale of the roughness. Similarly, at low frequencies, the transmission did not vary much with sample thickness. However, high-frequency phonons had a lower transmission as the SL became longer. Figure 3B shows the thermal conductivity of the SL versus length for samples with rough interfaces. Because the

transmission depended on the roughness conditions of the SL sample boundaries, which are difficult to predict due to the polycrystalline nature of the Al film, an exact match between experiments and simulation was not expected. Nevertheless, the temperature dependence of the thermal conductivity in the dominantly coherent regime ($T < 100$ K) was consistent between the experiments and the GF calculations. To obtain reasonable numerical agreement with experiments, the force constants between the Al layer and the SL were reduced and their masses were increased so as to artificially increase the interfacial thermal resistance by an order of magnitude. In actual experiments, it was found that this interfacial resistance depends highly on environmental and sample preparation conditions, which justifies such a treatment.

Our experimental and theoretical studies show that most of the phonons that contributed to the measured thermal conductivity in SLs traversed the SLs ballistically and hence were coherent. Interface roughness was effective in destroying the coherence of high-frequency phonons but not effective in scattering low-frequency phonons. The large reduction in thermal conductivity resulted from the loss of coherence of high-frequency phonons, but the lower-frequency phonons that contribute to the thermal conductivity were mostly coherent during their transport through the SL structures until they were scattered at the sample boundaries. This conclusion has important implications for the future of phonon engineering. For thermoelectric applications, for example, a lower thermal conductivity can be achieved if the coherence of these low-frequency phonons can be broken. The coherence of long-wavelength phonons can be destroyed by introducing perturbations that vary over long length scales. Examples of such perturbations are the strain associated with dislocations and aperiodic SLs. Indeed, some past experiments show that quantum-dot SLs (40), metal-dielectric SLs (41), and disordered, layered crystals (42) exhibit very low thermal conductivities that may have been the result of the destruction of long-wavelength phonon coherence. With the identification of the contribution of coherent phonons to heat conduction, we expect that strategies can be developed to further suppress or enhance the thermal conductivity of nanostructured materials.

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Materials and Methods
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References (43–57)

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Evidence for a Dynamo in the Main Group Pallasite Parent Body

John A. Tarduno,^{1,2*} Rory D. Cottrell,¹ Francis Nimmo,³ Julianna Hopkins,² Julia Voronov,¹ Austen Erickson,^{1,2} Eric Blackman,² Edward R.D. Scott,⁴ Robert McKinley¹

Understanding the origin of pallasites, stony-iron meteorites made mainly of olivine crystals and FeNi metal, has been a vexing problem since their discovery. Here, we show that pallasite olivines host minute magnetic inclusions that have favorable magnetic recording properties. Our paleointensity measurements indicate strong paleomagnetic fields, suggesting dynamo action in the pallasite parent body. We use these data and thermal modeling to suggest that some pallasites formed when liquid FeNi from the core of an impactor was injected as dikes into the shallow mantle of a ~200-kilometer-radius protoplanet. The protoplanet remained intact for at least several tens of millions of years after the olivine-metal mixing event.

Lord Rayleigh (Robert John Strutt) (1) noted the paradox posed by pallasite meteorites: Olivine and metal seemingly should have separated into layers in their parent body. Some models, to avoid segregation, have invoked small metal pools throughout a parent body (2), but the putative scenario has remained in formation near a core-mantle boundary (3). There are ~50 known pallasite meteorites. Most have isotopic ratios that fall near the terrestrial mass fractionation line and are called “main group” pallasites (4). Olivine ranges from Fa₁₁ to Fa₂₀ and often occurs as centimeter-sized (Fig. 1, A and B) crystals (5–8), with a dislocation density

(9) comparable with those of unshocked terrestrial samples. The metal in main group pallasites is Ir poor and is thought to have originated from the residual melt fraction of a core similar in composition to IIIAB iron meteorites (3).

Paleomagnetism might help to distinguish between models for pallasite formation, but prior attempts have failed to yield interpretable data. The massive FeNi of the pallasite matrix is the likely culprit. This metal is similar to that composing iron meteorites, which carries a highly anisotropic, soft magnetization; it is notoriously poor as a paleomagnetic recorder (10, 11). Paleomagnetic studies of other meteorites [for example, (12–13)], however, suggest some parent bodies hosted dynamos. Modeling suggests bodies >80 km in radius could be in the regime of supercritical magnetic Reynolds numbers, in which large-scale dynamo action is possible (14, 15).

Rather than studying bulk material, we applied techniques of single-silicate crystal analysis (16, 17) to an investigation of the Ilmälac and Esquel main group pallasites. We selected gem-like olivine subsamples ≥0.5 cm from the me-

teorite edge and several millimeters from the olivine/metal contact. Prior studies (18, 19) suggest that at these distances, heating effects due to atmospheric entry are negligible.

We have observed strings of large inclusions, tens of micrometers in size (Fig. 1C), in some olivines using transmitted light microscopy. Scanning electron microscopy (SEM) reveals isolated and strings of much smaller inclusions (≤10 μm) (Fig. 1D) that are composed of Fe, Ni, S, and Cr (fig S3). Microprobe analyses detail submicrometer-sized, irregularly spaced FeNi particles within these smaller inclusions, surrounded by troilite (fig S4). These metal particles are sometimes Ni rich [~51 to 58 weight percent (wt %) Ni] and are potential stable magnetic recorders.

Olivine subsamples lacking inclusions visible to the naked eye show pseudo-single- to single-domain magnetic hysteresis behavior (Fig. 1, E and F). In contrast, samples with visible inclusions have multidomain behavior. In the former case, we find only a slight anisotropy (Fig. 1G), and first-order reversal curves (20) fail to show substantial magnetic interactions (Fig. 1H). Thus, we further selected olivine subsamples lacking visible inclusions because they can have optimal properties for paleointensity determination (21).

Many meteorites have been exposed to magnetic contamination during collection (13). We therefore first used alternating field demagnetization, which revealed removal of magnetizations after the application of low peak fields (5 to 10 mT). Magnetization directions stabilized after this pretreatment, and it was here that we started thermal demagnetization. We used thermal methods because they best replicate the potential magnetization acquisition process [thermoremanent magnetization (TRM)] (21). In many meteorites, magnetic mineral alteration accompanying thermal treatment is severe (11–13). Studies of terrestrial samples indicate that inclusions in single-silicate crystals are less susceptible to alteration (16, 17).

¹Department of Earth and Environmental Sciences, University of Rochester, Rochester, NY 14627, USA. ²Department of Physics and Astronomy, University of Rochester, Rochester, NY 14627, USA. ³Department of Earth and Planetary Sciences, University of California, Santa Cruz, CA 95064, USA. ⁴Hawaii Institute for Geophysics and Planetology, University of Hawaii, Manoa, HI 96822, USA.

*To whom correspondence should be addressed. E-mail: john.tarduno@rochester.edu

Low unblocking temperature magnetizations (<360°C) observed from Esquel olivine likely have a viscous origin. However, the Esquel

pallasite olivine shows a large decrease in natural remanent magnetization (NRM) and a stable direction between ~360° and 500°C (Fig. 2,

A and B). Only very small NRM changes are seen at higher demagnetization temperatures, between 500° and 750°C. The dominant drop

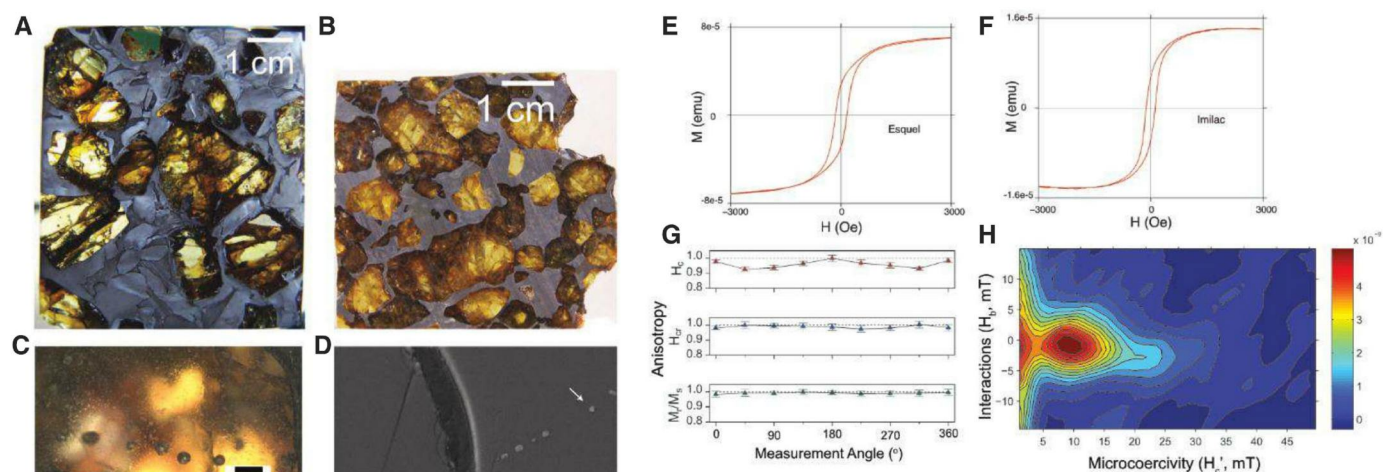


Fig. 1. Magnetic character of inclusions in pallasite olivine. (A and B) Esquel and Imilac meteorite samples, respectively. (C) Large inclusions in olivine (transmitted light microscopy). (D) String of smaller inclusions (between white arrows; SEM). (E and F) Magnetic hysteresis curves for olivine. (G) Hysteresis parameter versus angle of measurement (16) and (H) First-order reversal curve plot (20) for Esquel olivine. M_r , remanent magnetization; M_s , saturation magnetization; H_{cr} , coercivity of remanence; H_c , coercivity.

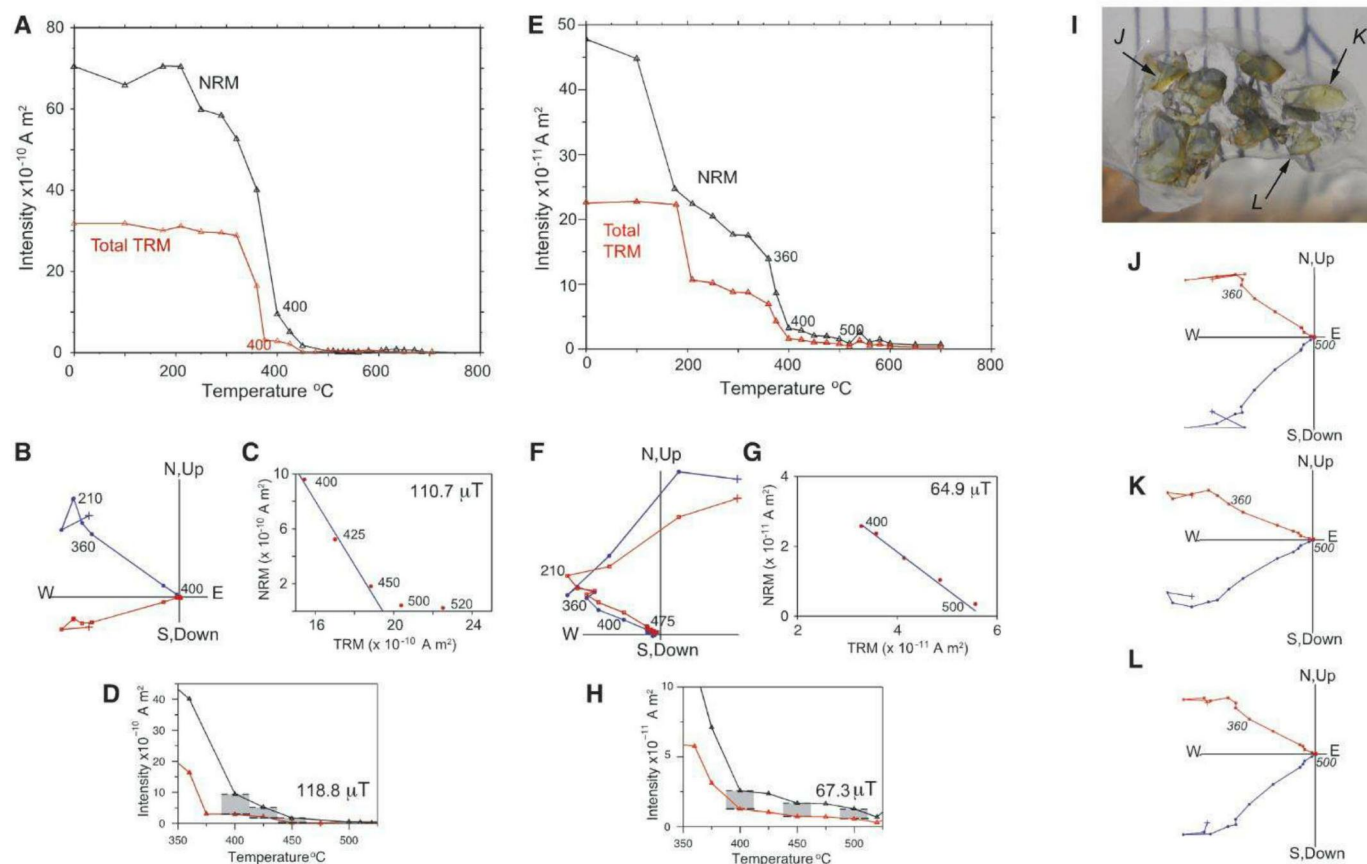


Fig. 2. Paleointensity experiments on pallasite olivine. (A) Demagnetization of NRM of Esquel olivine (black line). (B) Orthogonal vector plot of (A); red is inclination, blue is declination (orientation relative). (C) Thellier-Coe paleointensity data, NRM removed versus TRM gained using a 60- μ T applied field suggests a paleofield of 110.7 μ T. (D) Demagnetization of a laboratory Total TRM acquired in a 60- μ T field [(A), red curve] suggests a paleofield of 118.8 μ T

(calculated by comparing values at three temperature steps highlighted by gray boxes). (E to H) Paleointensity data as discussed above on Imilac olivine indicating paleofields of 64.9 μ T (Thellier-Coe technique, 60- μ T applied field) and 67.3 μ T (Total TRM method, 30- μ T applied field). (I) An oriented section of the Esquel meteorite with metal removed. (J to L) Associated demagnetization results.

in NRM suggests a taenite carrier (~ 50 to 55 wt % Ni) (21, 22), which is consistent with our microprobe results. Ordering may be limited in very small taenite particles within troilite inclusions (23).

Thellier-Coe (23, 24) paleointensity data (Fig. 2C) of a typical sample suggest that a TRM was imparted in a paleofield of 110.7 ± 5.2 μ T. To further examine the nature of the NRM, we imparted a Total TRM to the sample by heating at 700°C in the presence of a 60 - μ T field. The demagnetization curve of the Total TRM is similar to that of the initial NRM (Fig. 2A); small dif-

ferences may indicate minor thermally induced alteration. Demagnetization of the Total TRM allows for a second estimation of the paleofield; this yields 118.8 ± 5.7 μ T. Subsamples from two additional crystals from the same Esquel meteorite sample yield similar values (116.0 ± 5.4 μ T, 109.6 ± 7.0 μ T, Thellier-Coe method; 115.0 ± 6.9 μ T, 113.4 ± 4.0 μ T, Total TRM method). As a further consistency test, we studied a second Esquel pallasite sample. We observed nearly identical demagnetization behavior, with Thellier-Coe and Total TRM paleointensity estimates of 132.4 ± 5.7 μ T and 134.3 ± 6.1 μ T, respectively, within

$\sim 15\%$ of the values obtained from our first experiments (tables S1 to S4).

Olivine subsamples from the Imilac pallasite show similar behavior (Fig. 2, E to H). Thellier-Coe experiments on two separate samples yield 67.9 ± 9.2 μ T and 79.3 ± 7.2 μ T (paleointensities based on Total TRM experiments are 67.7 ± 6.2 and 77.7 ± 2.2 μ T, respectively). Total TRM experiments using two different applied field values yield consistent paleointensities (table S2), suggesting no applied field dependence.

The unblocking temperatures we have observed, viewed in the context provided by our microprobe results, are inconsistent with terrestrial weathering (23). Also, our experiments demonstrate that the dominant magnetization is not an artifact of kamacite-taenite interaction discussed in the study of iron meteorites (10). Our paleointensity measurements are on unoriented olivine crystals. In some meteorites, subsamples have been found to have different magnetic directions, precluding the acquisition of a TRM after the meteorite mass had assembled (11–13). In contrast, at unblocking temperatures $>360^\circ\text{C}$, we observed consistent directions from oriented pallasite olivine crystals (Fig. 2, I to L).

The average field value obtained from the Esquel meteorite (122.3 ± 14.4 μ T, Thellier-Coe method; 125.2 ± 12.9 μ T, Total TRM method) is somewhat larger than those observed on Earth's surface but somewhat weaker than Earth's field calculated at the core-mantle boundary (for example, the radial component was typically 200 to 600 μ T in 1990) (25). The average value from the Imilac meteorite (73.6 ± 8.1 μ T, Thellier-Coe method; 72.7 ± 7.1 μ T, Total TRM method) is comparable with Earth's surface field. These relatively high intensities suggest an internally generated magnetic field in the pallasite parent body because other sources create fields orders of magnitude weaker (13). We interpret these data as recording dynamo action after the injection of metal into the olivine crystals. The fracture pathways for the metal injection subsequently healed, and the inclusions cooled below the Curie temperature of taenite. This injection probably coincided with an impact creating the larger-scale olivine-metal mixing.

The absolute age of the mixing event is unknown, but Mn-Cr systematics provide an oldest age bound of 4.558 billion years ago (26). Fission-track model ages suggest that the magnetization we have measured may have set in as late as 4.4 to 4.2 billion years ago (27), values that are consistent with an early mixing event followed by slow cooling (23).

Our data thus imply that the parent body must have retained a partially liquid iron core (to permit a dynamo) until the pallasites cooled to $\sim 360^\circ\text{C}$, and therefore they cannot have been too close to the core-mantle boundary. The magnetic evidence is consistent with, and independent of, the diversity of main group pallasite cooling rates that previously have been used to argue (28) against a core-mantle boundary origin. A liquid

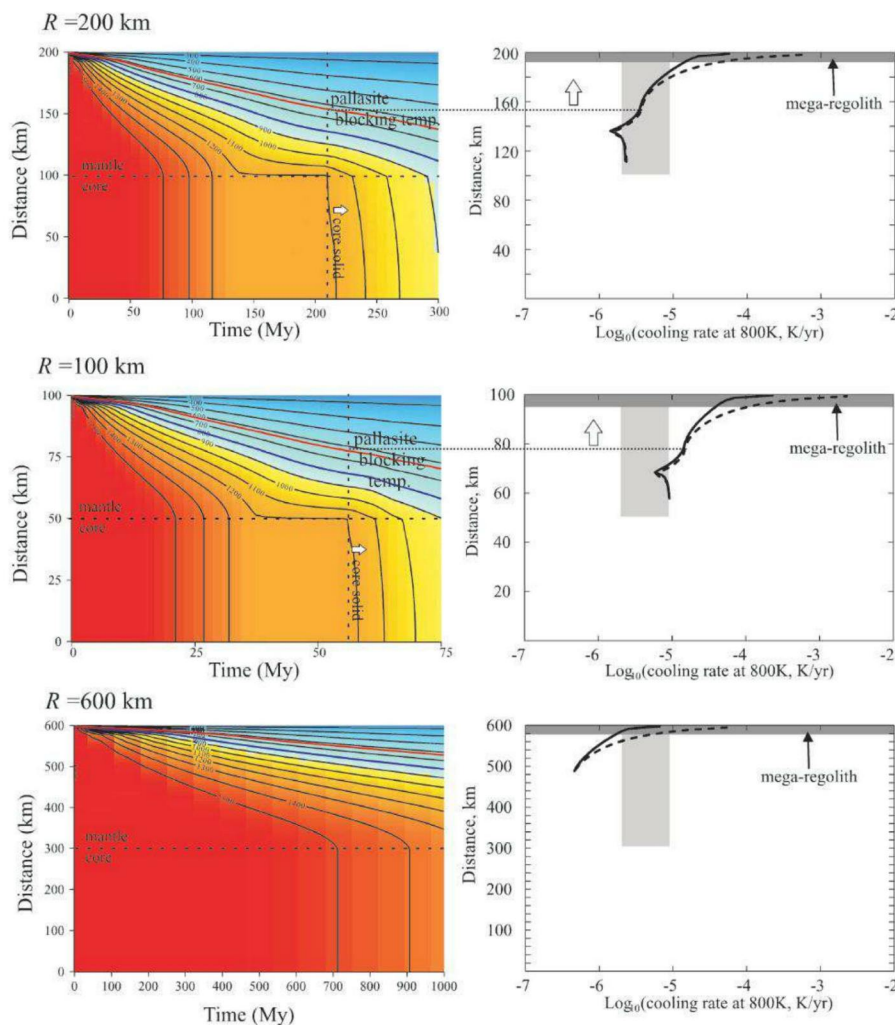


Fig. 3. Spherically symmetric three-layer conductive asteroid cooling model (23). **(Left)** Evolution of temperature as a function of radius and time. The model consists of an insulating regolith, a silicate mantle, and a metallic core. The initial condition is 1600 K everywhere. The core remains isothermal (liquid) until it starts to solidify at 1200 K and thereafter cools conductively. The mantle cools conductively throughout. The 800 K and 633 K isotherms correspond to taenite diffusion recording cooling rate and the lowest paleomagnetic unblocking temperature defining the characteristic magnetization, respectively. The horizontal dashed line indicates the core mantle boundary, and the vertical dashed line indicates the time at which core solidification is complete. **(Right)** Cooling rate at 800 K as a function of distance. The dark shaded box indicates the assumed megaregolith thickness (23). The light shaded box is the 2 to 9 K per million years cooling rate estimate from pallasite metal experiments (28). The solid and dashed lines represent model cooling rates with and without a megaregolith, respectively. The core was still convecting (not solid) when the pallasites reached 633 K. So, the pallasites must be shallower than the depth indicated by the dotted line. For a 200-km-radius body, there is a region at radius (r) ~ 160 km at which both the cooling rate and the paleomagnetic constraint are satisfied.

core requires a temperature exceeding ~ 1200 K (29), so assuming conductive cooling (23), the pallasites we have investigated were in the top $\sim 60\%$ of the protoplanet mantle. Cooling rates at 800 K (the diffusion temperature of taenite) in this depth range in a 200-km-radius body match estimated pallasite metal cooling rates (28) of 2 to 9 K per million years (Fig. 3). Conversely, in a larger 600-km-radius body the pallasites would have to have resided in the near-surface megaregolith, which is inconsistent with their unshocked state, whereas in a smaller 100-km-radius body, the cooling rate is too fast (Fig. 3). Compositional convection in the core (14) can drive the dynamo, and impacts can provide additional short-term stirring (30). For a 200-km-radius body, pressure effects on the magnetization are likely minor (23). These conclusions on parent body size assume the pallasites were not remagnetized during impact heating subsequent to the olive-metal mixing event. If such reheating occurred, parent bodies ranging from 100- to 200-km radius could satisfy the data, and the pallasites could have formed deeper in the parent body, within 10% of the core-mantle boundary. However, we view this as improbable because such reheating is inconsistent with the low observed pallasite shock state (23).

The factor of ~ 2 difference between Esquel and Ilmilac paleointensity estimates could indicate different positions within the protoplanet. For instance, the Esquel and Ilmilac meteorites could have resided at original depths of 40 km and 10 km, respectively, within a 200-km-radius body, assuming a dipolar field. In this case, the Curie isotherm of taenite would be reached at 180 million and 52 million years after the body formed for the Esquel and Ilmilac pallasites, respectively (Fig. 3). The heat fluxes at the core at these times are 33 and 0.8 mW m^{-2} , respectively; the former at least is sufficient to drive a dynamo if compositional convection occurs (14). However, the paleointensity difference could also be explained by a smaller difference in original depth com-

bined with a time-dependent dynamo field. In any event, generation of a strong, magnetic field by a dynamo at least several tens of millions of years after olivine/metal mixing is required by our data.

We recall that the pallasite metal is Ir poor, implicating a fractionated source. This requirement together with the likely position of the pallasites in the protoplanet and the time constraints on when the dynamo was active suggest that the pallasite metal was derived from the liquid iron core of a differentiated asteroid impactor (fig. S7) that struck before the Curie isotherm was reached. The metal could have been introduced into a dunite mantle as dike-like intrusions, similar to impact melt dikes seen in terrestrial impact structures (31). This mechanism provides a solution to the pallasite paradox because dikes propagating through relatively cold olivine will undergo an initial phase of rapid cooling, freezing in the olivine-metal pallasite structure, before cooling through the taenite Curie temperature. The differentiated pallasite parent body may have been formed in the terrestrial planet-forming zone (32). If so, the timing of dynamo action suggests that the pallasite protoplanet was one of the few, late survivors in this zone before a cataclysmic collision that scattered pallasite fragments from a position closer to the Sun outward to the asteroid belt.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S7

Tables S1 to S5

References (33–65)

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Evidence for Early Hafted Hunting Technology

Jayne Wilkins,^{1*} Benjamin J. Schoville,² Kyle S. Brown,^{2,3} Michael Chazan¹

Hafting stone points to spears was an important advance in weaponry for early humans. Multiple lines of evidence indicate that $\sim 500,000$ -year-old stone points from the archaeological site of Kathu Pan 1 (KP1), South Africa, functioned as spear tips. KP1 points exhibit fracture types diagnostic of impact. Modification near the base of some points is consistent with hafting. Experimental and metric data indicate that the points could function well as spear tips. Shape analysis demonstrates that the smaller retouched points are as symmetrical as larger retouched points, which fits expectations for spear tips. The distribution of edge damage is similar to that in an experimental sample of spear tips and is inconsistent with expectations for cutting or scraping tools. Thus, early humans were manufacturing hafted multicomponent tools $\sim 200,000$ years earlier than previously thought.

Behavioral traits common to both modern humans and Neandertals could represent shared traits inherited from their

last common ancestor, commonly held to be *Homo heidelbergensis* (1, 2). The fossil record for *H. heidelbergensis* begins during the early

Middle Pleistocene, and genetic studies situate the divergence of *H. sapiens* and Neandertal lineages at between ~ 800 and 400 thousand years ago (ka) (3). Because Middle Stone Age (MSA) hominins and Neandertals probably both had stone-tipped hunting equipment, it is possible that *H. heidelbergensis* also possessed this form of technology.

By ~ 780 ka, hominins were regularly killing large game, based on evidence of repeated in situ processing of complete carcasses of fallow deer at Gesher Benot Ya'kov in Israel (4). At the English

¹Department of Anthropology, University of Toronto, 19 Russell Street, Toronto, Ontario M5S 2S2, Canada. ²Institute of Human Origins, School of Human Evolution and Social Change, Post Office Box 872402, Arizona State University, Tempe, AZ 85287-4101, USA. ³Department of Archaeology, University of Cape Town, Rondebosch 7701, South Africa.

*To whom correspondence should be addressed. E-mail: jayne.wilkins@utoronto.ca

site of Boxgrove, a horse scapula with a semi-circular perforation is consistent with spear-aided hunting by ~500 ka (5). Wooden spears dating to ~400 ka have been found in association with butchered horses at Schöningen, Germany (6). Hafted spear tips appear to be common in the MSA and Middle Paleolithic (MP) sites of Europe and Africa after ~300 ka (7–20).

Here, we analyze lithic points recovered from stratum 4a at Kathu Pan 1 (KP1) in South Africa and show that these points were likely hafted onto the ends of spears. KP1 is located 4.5 km northwest of the town of Kathu, just west of the Kuruman Hills in the Northern Cape Province (fig. S1). The site is an infilled sinkhole with more than 5 m of Earlier Stone Age (ESA), MSA, and Later Stone Age (LSA) deposits identified in five geological strata (21–24).

Stratum 4a yields lithic artifacts that have been attributed to the Fauresmith Industry, based on the co-occurrence of blades, unifacial points, Levallois

cores, and handaxes (21–23). The Fauresmith is generally considered a late ESA, early MSA, or transitional industry. Two chronometric methods situate the KP1 stratum 4a lithic assemblage in the early Middle Pleistocene ~500 ka, coeval with *H. heidelbergensis* (25) and the genetic divergence of *H. sapiens* and Neandertals (3). When dated with optically stimulated luminescence (OSL), a sample taken from sediments in direct association with stratum 4a lithic artifacts gives an age estimate of 464 ± 47 ka, and an *Equus capensis* tooth recovered adjacent to the OSL sample gives a U-series/ESR age of 542^{+140}_{-107} ka (23, 26). Stratum 4a is truncated at the top by a sharp contact with overlying stratum 3 (26). An OSL sample from stratum 3, which yields MSA artifacts, gives an age estimate of 291 ± 45 ka and provides additional chronological control for stratum 4a (23).

The stratum 4a assemblage contains numerous unifacially retouched points (Fig. 1 and fig.

S3) and nonretouched triangular flakes and blades (fig. S4). The analyzed sample from four square units of KP1 contains 210 points and point fragments (26) (table S1). Most of these points are manufactured on banded ironstone and range from 28 to 123 mm in maximum length (mean = 70 ± 1.8 mm, table S2).

Some of the KP1 points have fractured tips, bases, or lateral edges, known as diagnostic impact fractures (DIFs) (Fig. 2, A to D). Similar features are seen in experiments in which weapon tips strike targets (27). Finding such fractures archaeologically implies that the points were used as weapon tips (11, 18, 20, 28–31). Similar-appearing fractures can result from post-depositional processes, although their frequency within assemblages is low (32). The frequency of KP1 points with DIFs is 13.8% [29 out of 210 (29/210), 95% confidence interval 9.8 to 19.2%], which is higher than expected for post-depositional processes (Fig. 2E, $\chi^2 = 45.532$, $df = 1$, $P < 0.001$) and consistent with frequencies observed in well-established weapon tips from Holocene residential sites (Fig. 2E, $\chi^2 = 1.337$, $df = 1$, $P = 0.287$). Impact experiments with similar points made of banded ironstone also exhibit these diagnostic fractures (8/32, 25.0%) (26).

About 13.0% (23/177, excluding distal fragments) of the KP1 points (fig. S5) show evidence of modifications near their bases. Typically, between two and seven flakes were removed from the base of each basal-modified KP1 point. This working could reflect intentional removals to shape the point to allow hafting. The frequency of basal modification is similar to those reported for MSA assemblages with evidence for hafted spear tips (20, 33).

To test the feasibility of KP1 points as spear tips, we reproduced points from the same raw material as most of the KP1 points (banded ironstone) and hafted them onto wooden dowels. We then thrust them into two culled springbok carcasses, using a calibrated crossbow to simulate a thrusting spear and keep force consistent (26). These points performed well and adequately penetrated the target. Most of the 32 replicated points had to be shot multiple times before exhibiting any visible damage, and only two trials resulted in catastrophic damage that prevented the reuse of the points (table S5).

Overall, the sizes of the KP1 points are similar to those of MSA hafted points. The tip cross-sectional perimeter (TCSP) has been used to approximate the size of the wound that would be created by points (17). KP1 points have TCSP values slightly larger than but overlapping with those of MSA points that have been interpreted as spear tips (Fig. 3A). KP1 points are much larger than arrow or dart tips.

Stone tools used for cutting become less symmetrical as one edge is preferentially resharpened; thus, small points are expected to be less symmetrical than large points in an archaeological assemblage of points used mainly as cutting or scraping tools (34). In contrast, spear tips are

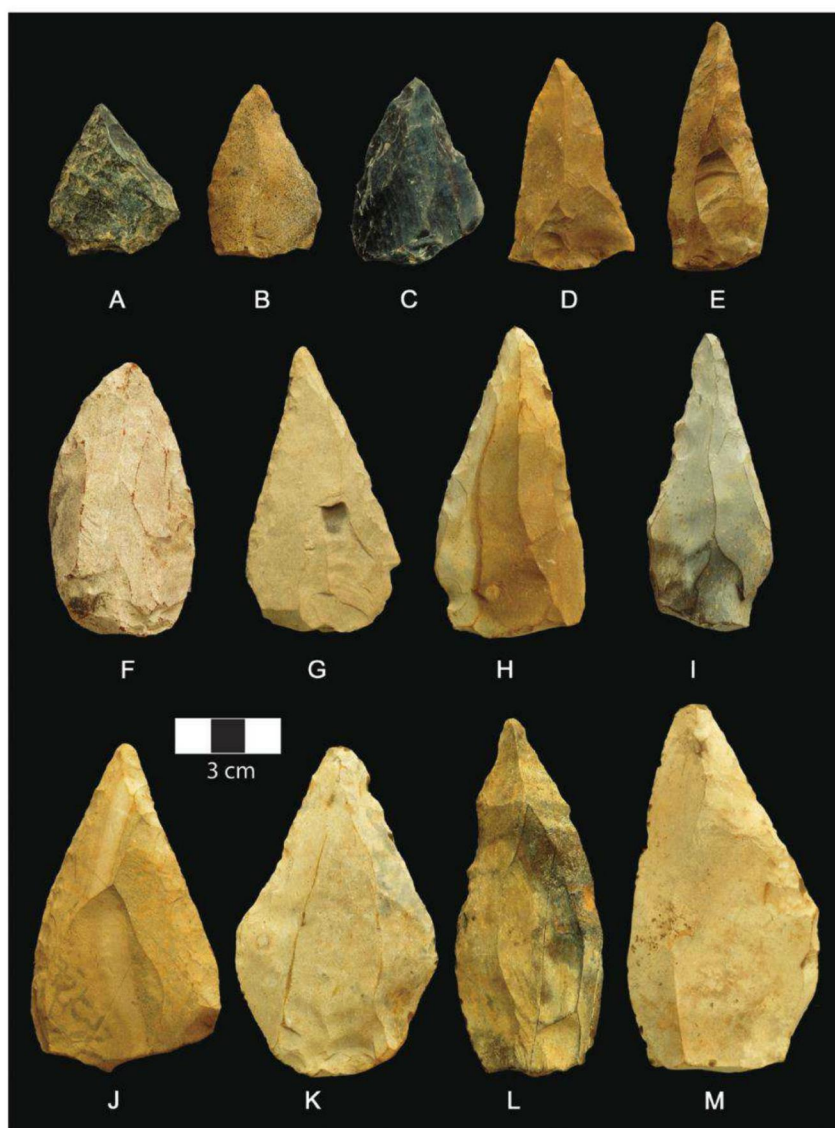


Fig. 1. (A to M) A sample of KP1 complete retouched points. All are banded ironstone except (A) and (C) (black chert). Additional points are presented in figs. S3 and S4.

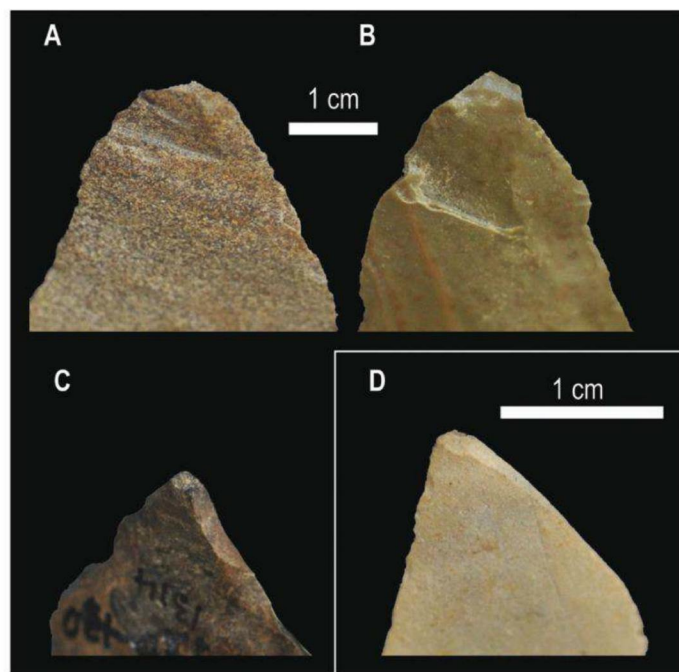
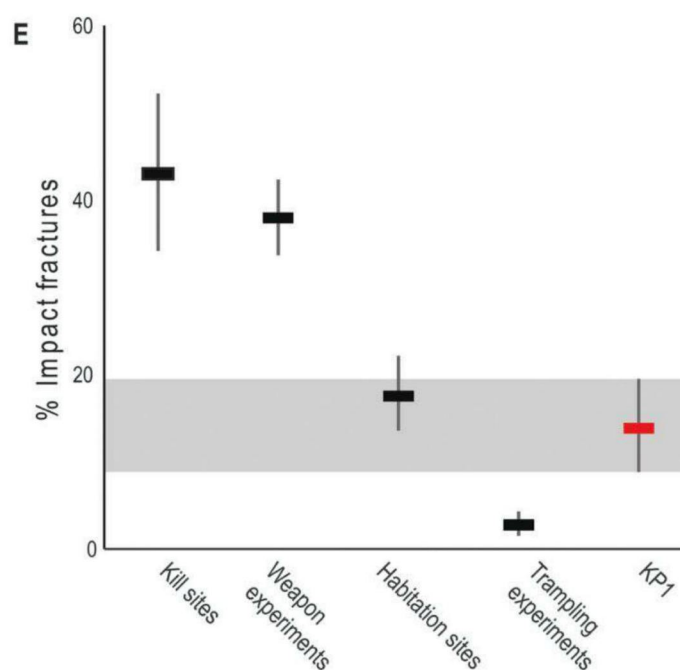
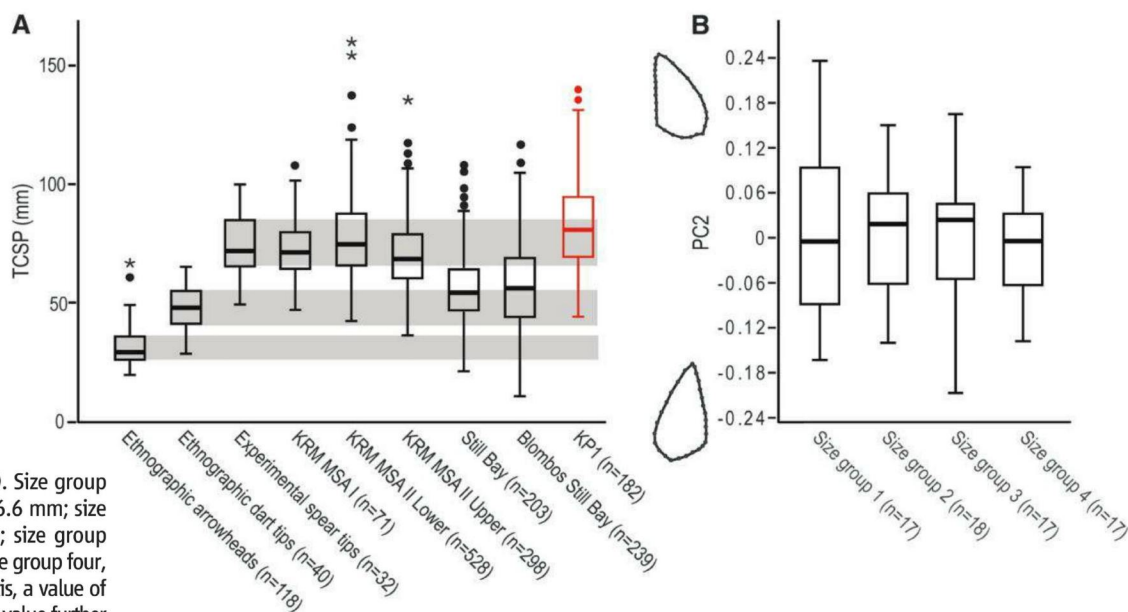


Fig. 2. (A and B) Distal step-terminating bending fractures on ventral surfaces of complete nonretouched convergent blades, banded ironstone. (C) Distal impact burination on ventral surface of a complete nonretouched convergent flake, banded ironstone. (D) Distal impact burination on ventral surface of



complete nonretouched convergent blade, banded ironstone. (E) Comparison of DIF frequencies (95% confidence intervals) at Holocene kill and habitation sites with well-established weapon tips (11, 27, 28, 36), experimental weapon tip studies (10, 27, 37–40), trampling experiments (32, 41), and KP1.

Fig. 3. (A) Box-plot comparison of published TCSP values for ethnographic arrowheads and darts (17), experimental spear tips (this study), MSA points from Klasies River Mouth (KRM), various South African Still Bay sites and Still Bay levels at Blombos Cave (17), and KP1 points (this study). (B) Box plot of principal component 2 (PC2) from the relative warps analysis (26) for four size groups (based on maximum length) of approximately equal sample size for all retouched KP1 points ($n = 69$). Size group one includes points 41.3 to 56.6 mm; size group two, 57.4 to 68.7 mm; size group three, 68.8 to 82.5 mm; and size group four, 82.8 to 117.8 mm. On the y axis, a value of zero represents symmetry, and a value further from zero represents decreased symmetry.



expected to be symmetrical regardless of size (26). There are no significant differences in the symmetry (analysis of variance, $F = 0.197$, $P = 0.898$) or variance (Levene's test, $P = 0.236$) of KP1 points grouped by size (Fig. 3B). Small retouched points are as symmetrical as large retouched points.

The distribution of damage along the edges of the KP1 points further supports their use as spear tips (Fig. 4). Using a low-powered micro-

scope, we recorded the macroscopic edge damage that was evident on all complete points (26). Edge damage was more frequent at point tips than along point edges, and distributions were similar between left and right sides (Fig. 4A).

Taphonomic processes can be ruled out as the sole source of damage on the KP1 points. Post-patination scars, which are easily identified (fig. S8D), reflect damage not related to use of the points and occur as frequently on point edges as

point tips (Fig. 4B). In aggregate, the damage along the dorsal surface of the KP1 points was similar to the distributions of post-patination scars [two-sample Kolmogorov-Smirnov tests (D , Kolmogorov-Smirnov statistic) were used for all comparisons of damage distribution; left, $D_{\max} = 0.060$, $D_{\text{obs}} = 0.039$, $P > 0.05$; right, $D_{\max} = 0.068$, $D_{\text{obs}} = 0.036$, $P > 0.05$], whereas the damage along the ventral surface was different (left, $D_{\max} = 0.060$, $D_{\text{obs}} = 0.087$, $P < 0.05$;

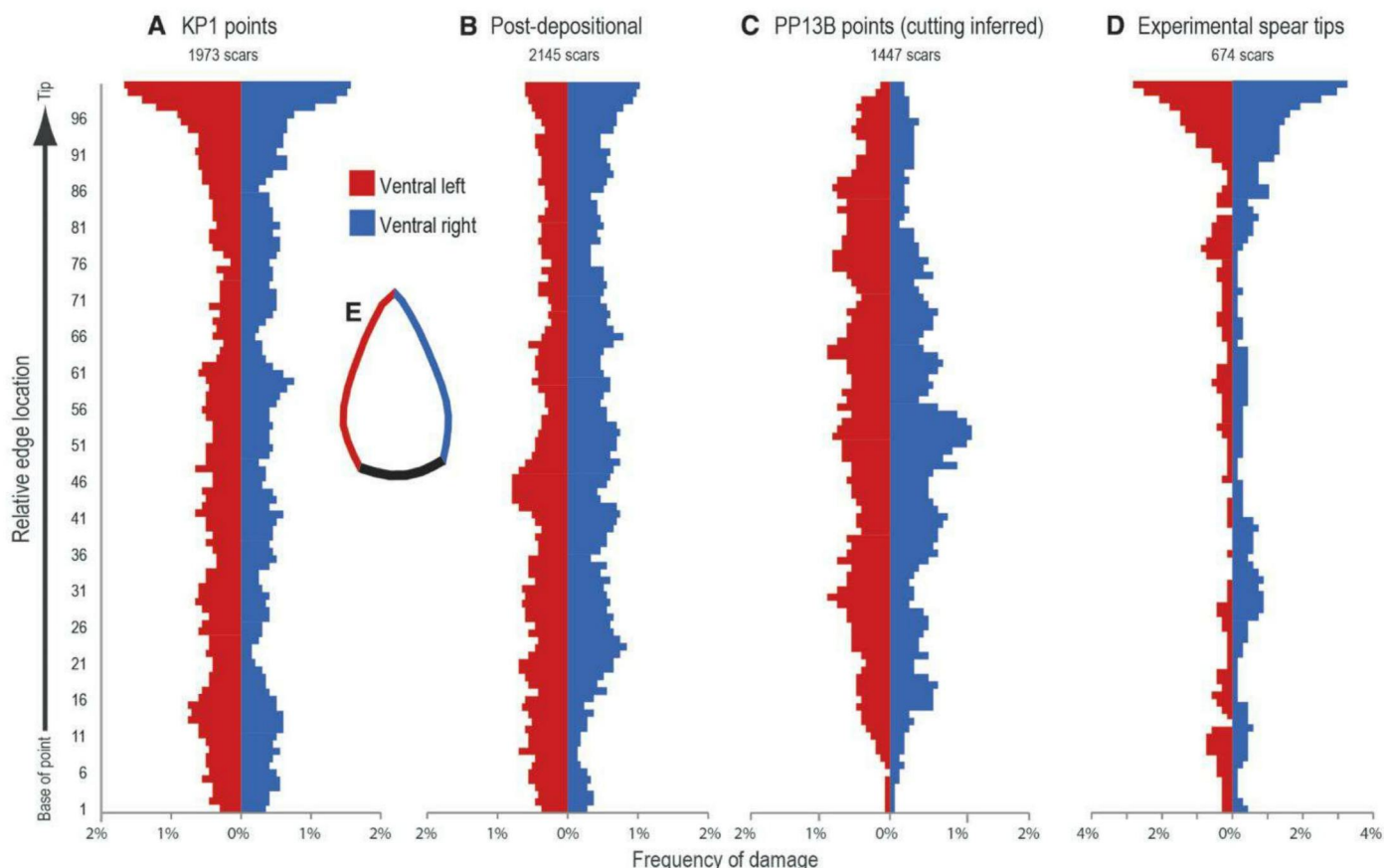


Fig. 4. Comparison of ventral edge damage distributions. **(A)** Complete KP1 points ($n = 106$). **(B)** Post-patination damage on complete KP1 points ($n = 106$), serving as proxy for post-depositional processes. **(C)** PP13B complete points, whose cutting function is inferred ($n = 86$). **(D)** Experimental spear tips ($n = 32$). **(E)** Consensus shape of KP1 points based on geometric morphometric analysis (26).

right, $D_{\max} = 0.060$, $D_{\text{obs}} = 0.114$, $P < 0.05$). Therefore, we focused on the ventral edge damage to test hypotheses about the function of KP1 points.

Ventral edge damage on the KP1 points is inconsistent with published edge damage on points from the MSA site Pinnacle Point Cave 13B (PP13B) in South Africa, which have been interpreted as cutting tools (35). At PP13B, there is no increase in damage at the tip (Fig. 4C), and the ventral left and right sides have statistically different damage distributions ($D_{\max} = 0.072$, $D_{\text{obs}} = 0.112$, $P < 0.05$). In contrast, the KP1 points exhibit increased damage at the tip, and the ventral left and right sides of the KP1 points are not statistically different ($D_{\max} = 0.0613$, $D_{\text{obs}} = 0.0610$, $P > 0.05$).

The KP1 edge damage distribution is consistent with spear tip expectations derived from our experimental sample of 32 replicated points (26). The experimental points exhibited increased damage at the tips (Fig. 4D), and the left and right sides were not statistically different ($D_{\max} = 0.106$, $D_{\text{obs}} = 0.084$, $P > 0.05$).

Multiple lines of evidence thus support the hypothesis that the KP1 points were used as spear tips. Evidence for hafted hunting technologies ~500 ka is consistent with the evidence that both

Neandertals and MSA hominins used hafted hunting tools and implies that this knowledge was also held by their common ancestor.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/338/6109/942/DC1
 Materials and Methods
 Figs. S1 to S8
 Tables S1 to S5
 References (42–53)

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Financial Costs of Meeting Global Biodiversity Conservation Targets: Current Spending and Unmet Needs

Donal P. McCarthy,^{1,2} Paul F. Donald,² Jörn P. W. Scharlemann,^{3,4} Graeme M. Buchanan,² Andrew Balmford,⁵ Jonathan M. H. Green,^{5,6} Leon A. Bennun,¹ Neil D. Burgess,^{5,7,8} Lincoln D. C. Fishpool,¹ Stephen T. Garnett,⁹ David L. Leonard,^{10*} Richard F. Maloney,¹¹ Paul Morling,² H. Martin Schaefer,¹² Andy Symes,¹ David A. Wiedenfeld,¹³ Stuart H. M. Butchart^{1†}

World governments have committed to halting human-induced extinctions and safeguarding important sites for biodiversity by 2020, but the financial costs of meeting these targets are largely unknown. We estimate the cost of reducing the extinction risk of all globally threatened bird species (by ≥ 1 International Union for Conservation of Nature Red List category) to be U.S. \$0.875 to \$1.23 billion annually over the next decade, of which 12% is currently funded. Incorporating threatened nonavian species increases this total to U.S. \$3.41 to \$4.76 billion annually. We estimate that protecting and effectively managing all terrestrial sites of global avian conservation significance (11,731 Important Bird Areas) would cost U.S. \$65.1 billion annually. Adding sites for other taxa increases this to U.S. \$76.1 billion annually. Meeting these targets will require conservation funding to increase by at least an order of magnitude.

After the failure of previous global commitments to reduce the rate of loss of biodiversity (1), parties to the Convention on Biological Diversity (CBD) recently adopted a new strategic plan, including 20 targets to be met by 2020 (2). Negotiations on financing the plan are not yet resolved, partly for lack of information on financial costs. We used data on birds, the best known class of organisms, to assess the financial costs of meeting two of the targets

relating to conserving species and sites: (i) preventing the extinction of known threatened species and improving and sustaining their conservation status (Target 12) and (ii) effectively managing and expanding protected areas to cover 17% of terrestrial and inland water areas (and 10% of coastal and marine areas), “especially areas of particular importance for biodiversity” (Target 11) (2). These two targets align closely with the existing focus of much of the conservation sector; they are also among the most immediately urgent, involving discrete actions amenable to costing.

To assess the costs of species conservation, we sampled 211 globally threatened bird species [19% of all threatened bird species on the International Union for Conservation of Nature (IUCN) Red List (3)]. We asked experts on each species to estimate (i) recent expenditure on conservation actions, and (ii) a range of costs for conservation actions needed to achieve the minimum improvement in status necessary to reclassify (“downlist”) each species to the next lowest category of extinction risk on the Red List (e.g., from Critically Endangered to Endangered). We modeled midrange cost estimates as a function of breeding distribution extent, degree of forest dependence, mean Gross Domestic Product per km² of breeding range states, and mean Purchasing Power Parity of breeding range states, and we used this model to estimate costs for all other globally threatened bird species (4) (fig. S1).

The median modeled annual cost per species for conservation actions required to achieve downlisting within 10 years was U.S. \$0.848 million (range: U.S. \$0.0387 to \$8.96 million; all values adjusted to 2012 U.S. \$) (Fig. 1A and table S1). This compares with a median of U.S. \$0.219 million annually [range: U.S. \$0.001 to \$4.82 million, standardized to the same 10-year period and adjusted for inflation (4)] for 25 threatened species that were successfully downlisted during 1988–2008 because of genuine improvements in their status (i.e., directly resulting from conservation interventions) (5) (table S2). Costs for all but one of these species fell within the range of our sample of estimated costs (Fig. 1A), although the median was significantly lower [analysis of variance (ANOVA) of natural log-transformed values: $F_{1, 259} = 7.4$, $P < 0.01$]. This may simply be because conservationists often prioritize species with more tractable conservation needs (6) or because, relative to all globally threatened birds, a disproportionate number of those 25 species are found on oceanic islands (76 versus 35%; $\chi^2 = 16.2323$, $df = 1$, $P < 0.001$), thus tending to have smaller ranges and hence lower costs.

Assuming that the actions required for each species are independent, we estimate the total costs of downlisting 1115 globally threatened bird species to be U.S. \$1.23 billion (U.S. \$0.975 to \$1.56 billion) annually over the next decade, excluding the costs of at-sea actions (4) (table S3). The estimated cost per species is <U.S. \$3 million annually for 95% of species (<U.S. \$1 million annually for 50%), and is lower for species in higher categories of extinction risk (Fig. 1B, ANOVA, $F_{2, 1112} = 74.4$, $P < 0.0001$) because they generally have smaller distributions. However, most costs are for actions (e.g., site protection) that will probably benefit other species whose distributions overlap; only 20% are for species-specific actions such as captive breeding. We therefore attempted to estimate the effects of such cost-sharing through a spatial analysis (4), which produced a revised minimum total of U.S. \$0.875 billion annually, of which U.S. \$0.379 to \$0.614 billion (43 to 49%) is needed in lower-income countries [low- and lower-middle-income countries as classified by The World Bank (4)]: those with greatest need for funding assistance (Table 1 and Figs. 2 and 3).

Investment of such sums does not guarantee success, as multiple factors (both deterministic and stochastic) may influence conservation outcomes (7, 8). Furthermore, many of these species will almost certainly require continued (and

¹BirdLife International, Wellbrook Court, Cambridge CB3 0NA, UK. ²RSPB, The Lodge, Sandy, Bedfordshire, SG19 2DL, UK.

³United Nations Environment Programme World Conservation Monitoring Centre, 219 Huntingdon Road, Cambridge CB3 0DL, UK. ⁴School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, UK. ⁵Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK. ⁶Woodrow Wilson School of Public and International Affairs, Princeton University, Princeton, NJ 08544, USA. ⁷Center for Macroecology, Evolution and Climate, Department of Biology, University of Copenhagen, DK-1165 Copenhagen, Denmark. ⁸Conservation Science Program, World Wildlife Fund, Washington, DC 20090, USA. ⁹Research Institute for the Environment and Livelihoods, Charles Darwin University, Northern Territory 0909, Australia. ¹⁰Pacific Cooperative Studies Unit (University of Hawai'i at Manoa), Hawaii Division of Forestry and Wildlife, Honolulu, HI 96822, USA. ¹¹Science and Technical Group, Department of Conservation, Christchurch, New Zealand. ¹²University of Freiburg, Faculty of Biology, Hauptstrasse 1, D-79104 Freiburg, Germany. ¹³12 Fishback Court, Warrenton, VA 20186, USA.

*Present address: U.S. Fish and Wildlife Service, Portland, OR 97266, USA.

†To whom correspondence should be addressed. E-mail: stuart.butchart@birdlife.org

possibly even increased) funding to maintain any improvement in their status beyond 2020, particularly given the likely intensification of existing threats, the increasing impacts associated with climate change, and the emergence of potential new threats (9).

The median annual expenditure within the last decade for the 211 species in our sample was U.S. \$0.065 million (range: \$0 to \$15.2 million), with the majority of resources spent on just a few species, which reflects a common pattern documented at national levels (10–12). This covered a median of 12% of the estimated required annual

expenditure per species. Recent funding was adequate (>90% of estimated need) for only 3% of species ($n = 7$), and <50% of required expenditures were covered for 86% of species. Extrapolation suggests that, to cover the U.S. \$0.875 to \$1.23 billion annually required to meet the CBD target for birds, an additional U.S. \$0.769 to \$1.08 billion per year is needed (but only U.S. \$0.314 to \$0.509 billion, 41 to 47% in lower-income countries) (Table 1 and Fig. 2). Given that the species for which we could obtain data may be biased toward those that are already receiving funding, the true shortfall may be even greater.

Conservation costs per species appear to be lower for other taxa (except possibly mammals), presumably because they have smaller distributions on average than birds (tables S4 and S5). Data for 664 declining threatened species in New Zealand (63 birds, 601 mammals, amphibians, reptiles, fish, invertebrates, vascular plants, bryophytes, and fungi) show that annual costs for birds are 4.20 times larger than the median for other taxa (4). Threatened birds make up 7.65% of all threatened species on the global IUCN Red List (4, 13), which suggests that the total annual costs of conserving all “known threatened species” as called for in the CBD target (2) by downlisting them by ≥ 1 Red List category may range from \$3.41 billion (if the proportion of costs that are shared among birds is the same for all other taxa) to \$4.76 billion (if one assumes no cost-sharing).

To estimate the costs of meeting the CBD target for site conservation, we carried out a separate analysis to quantify the costs of effectively conserving all terrestrial Important Bird Areas (IBAs). IBAs represent the largest systematically identified global network of important sites for biodiversity (14), as they make up 11,731 sites supporting populations of one or more of 4445 threatened, restricted-range, biome-restricted or congregatory species (15). Only 28% of IBAs are completely covered by existing protected areas, 23% are partially protected, and 49% are entirely unprotected (14). Protection [encompassing all types of protected area management and governance (16)] of all unprotected and partially protected IBAs (32% of which are in lower-income countries) would increase terrestrial protected area coverage to 17.5% and meet the CBD site target (14).

We estimated the costs of effectively managing IBAs by modeling required expenditure per hectare as a function of socioeconomic and site-specific variables (4), on the basis of data for 396

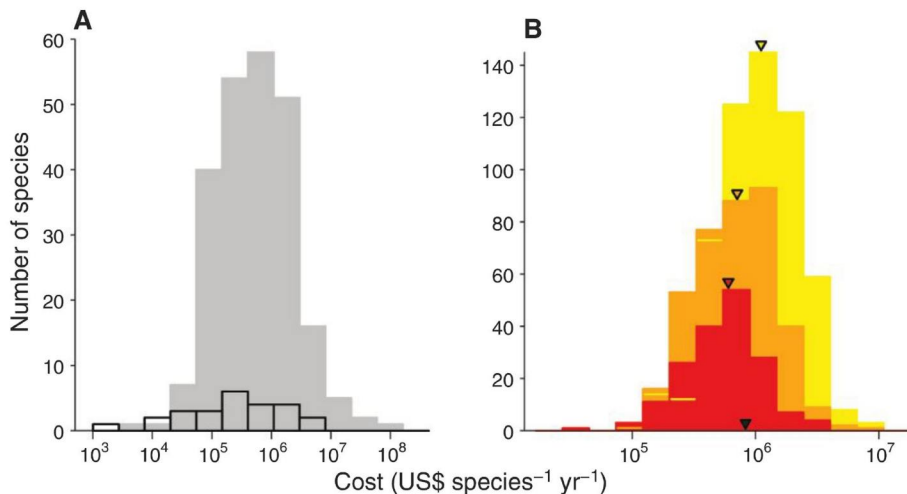


Fig. 1. (A) Estimated annual financial costs of conservation actions needed to downlist 211 globally threatened bird species to lower categories of extinction risk on the IUCN Red List within 10 years (solid bars), compared with actual costs of actions that led to successful downlisting of 25 species during 1988–2008 [outlined bars; corrected to the same 10-year period and adjusted for inflation (4)]. (B) Modeled annual costs per species of conservation actions needed to downlist 174 Critically Endangered species (red), 380 Endangered species (orange) and 561 Vulnerable species (yellow bars); horizontal colored lines indicate the height of obscured bars; arrows show medians (black indicates median across all 1115 bird species).

Table 1. Global costs of bird species conservation and site protection and management (billion U.S. \$ per year over the next 10 years; figures in parentheses give the % of the global total in each income group). Low-income and lower-middle-income countries are referred to in the text as “lower-income” countries; high-income and upper-middle-income countries

are referred to as “higher-income countries.” Threatened bird species excludes taxa listed as Possibly Extinct or Vulnerable under criterion D2 (4). Minimum values for column 5 are the costs of effectively managing additional sites; maximum values include costs of expanding protected areas.

Location	Preventing extinctions and conserving species ($n = 1115$ threatened bird species)		Protecting and managing sites ($n = 11,731$ IBAs)			
	Total required	Current shortfall	Effective management of existing protected sites	Current shortfall for existing protected sites	Establishing and managing additional protected sites	Total required
High-income countries	0.190–0.220 (18–22%)	0.159–0.184 (17–21%)	4.69 (65%)	2.49 (64%)	3.54–24.2 (42–50%)	28.9 (44%)
Upper-middle-income countries	0.305–0.407 (33–35%)	0.289–0.386 (35–38%)	0.907 (13%)	0.332 (8%)	1.88–16.01 (26–28%)	16.9 (26%)
Lower-middle-income countries	0.320–0.527 (37–42%)	0.264–0.435 (34–40%)	1.29 (18%)	0.867 (22%)	1.34–12.7 (19–22%)	14.0 (22%)
Low-income countries	0.0594–0.087 (7%)	0.0501–0.074 (7%)	0.293 (4%)	0.221 (6%)	0.354–4.89 (5–8%)	5.19 (8%)
Global	0.875–1.24*	0.769–1.08	7.18	3.91	7.11–57.8	65.1

*Note rounding errors explain the difference between the maximum and the 1.23 quoted in the main text.

sites across 50 countries (table S6). Extrapolation suggests that the total cost worldwide would be U.S. \$7.18 billion annually for currently protected IBAs, of which U.S. \$1.58 billion (22%) is required in lower-income countries.

We assessed the costs of expanding protected area networks to cover all unprotected and partially protected IBAs using spatially explicit agricultural land values (gross economic rents) from Naidoo and Iwamura (17) as a proxy for purchase or compensation costs (4). This produced a total cost of U.S. \$50.7 billion annually (U.S. \$15.9 billion, 31%, in lower-income countries), which is comparable to previous estimates of protected area expansion costs in developing countries (18). Applying our management cost model (see above) to these sites yields an estimate of U.S. \$7.11 billion annually, resulting in a total figure of U.S. \$57.8 billion required annually for protecting and effectively managing all IBAs.

Globally important sites have also been systematically identified for mammals; amphibians; and certain reptile, fish, plant and invertebrate groups in 12 countries (19, 20). Of these sites, 71% already qualify as IBAs and cover 80% of the total area (14). Assuming the areal relation holds worldwide and that such sites have a level of protection similar to that of IBAs, we estimate that protecting and effectively managing a more taxonomically comprehensive global network of terrestrial sites would cost U.S. \$76.1 billion annually (U.S. \$22.4 billion annually, 29% in lower-income countries) (Fig. 2).

We estimate that current annual expenditure on managing IBAs that are already under some form of protection falls short of requirements by

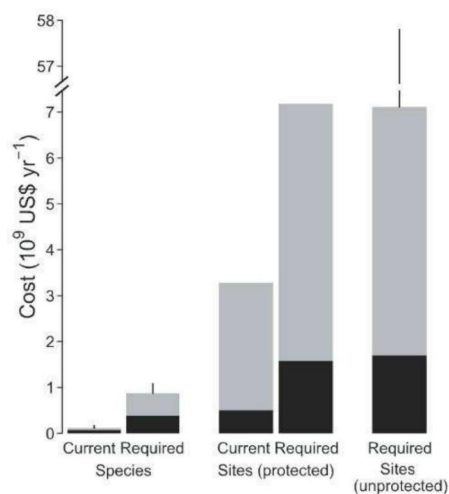


Fig. 2. Current expenditure and total required for conserving 1115 threatened bird species and safeguarding 11,731 important sites for birds in lower- and higher-income countries (black and gray bars, respectively). Bars for species show costs accounting for sharing between species, with vertical lines indicating costs excluding sharing. Bars for sites indicate management costs, with the vertical line for unprotected sites showing acquisition costs.

U.S. \$1.09 billion annually in lower-income countries (31% of needs covered) and by \$2.82 billion annually in higher-income countries [50% of needs covered, although this figure is based on more limited data (4)]. Management of an ex-

panded protected area network covering all currently unprotected or partially protected IBAs increases the estimated shortfall to \$2.78 billion for lower-income countries and \$8.24 billion for higher-income countries.

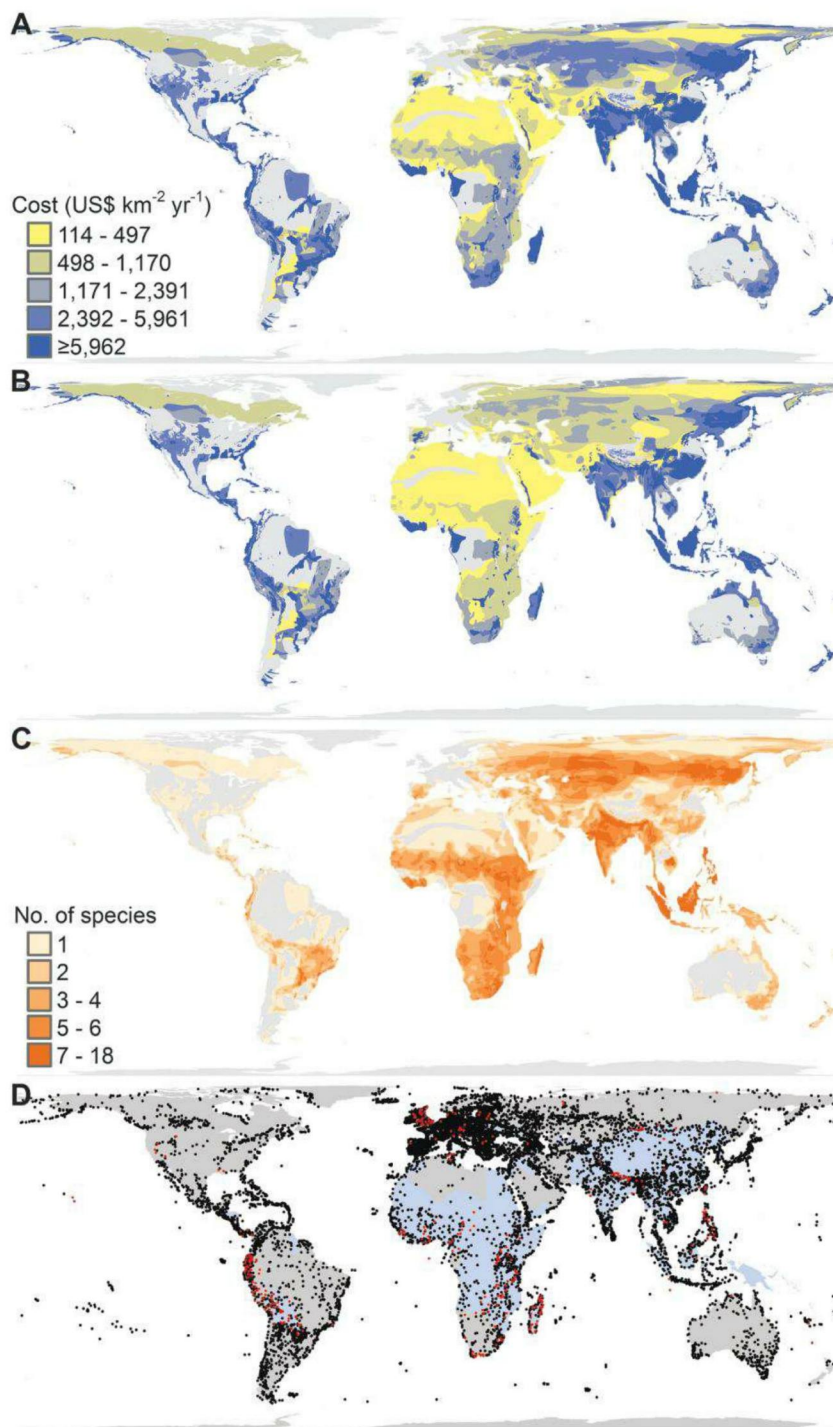


Fig. 3. Geographic patterns in the annual cost for conservation actions (U.S. \$ km⁻²) for 1097 globally threatened bird species (A) assuming that actions for each species are independent and (B) assuming cost sharing; (C) number of species km⁻², and (D) distribution of IBAs (points; red indicating those for which management-cost data were included in the model) and lower-income countries (blue shading). Costs and number of species are divided into quantiles; areas with no globally threatened bird species present shown in gray in (A to C); Behrmann equal-area projection. Distribution maps for 18 globally threatened species are not available.

A proportion of the costs would be shared between the species and site targets considered here. Establishing protection and managing sites made up 50 to 55% of the total costs for sampled bird species. Discounting this proportion from the total cost of species conservation across all taxa, a combined cost needed to meet both species and site CBD targets may be in the order of U.S. \$78.1 billion annually (Fig. 2). It is also highly likely that actions to meet these two targets will contribute to other targets in the CBD strategic plan, which are critical to delivering sustainable development and the safeguarding of global biodiversity in the long term (4).

Even with increased investment, careful prioritization will continue to be necessary to inform decisions about which areas to protect and which actions to undertake for species, e.g., using approaches that optimize returns on investment, given fixed budgets and defined objectives, for sites (21), species (7, 8), and management actions (22). Our finding that species facing higher categories of extinction risk require less investment for downlisting than do those in lower categories suggests that in many cases such analyses will prioritize actions for the most-threatened species first. We also note that there is considerable global spatial variation in costs and the number of threatened species per unit area (Fig. 3). Although the shortfalls in higher-income countries are substantial, the greatest gains per dollar will be in lower-income countries (23).

Despite the limitations of the available data, the shortfalls we have identified clearly highlight the need to increase investment in biodiversity conservation by at least an order of magnitude, especially given the small, but growing, body of evidence linking spending and effectiveness (24, 25). Nevertheless, the total costs are small relative to the value of the potential goods and services that biodiversity provides (26), e.g., equivalent to 1 to 4% of the estimated net value of ecosystem services that are lost per year [estimated at \$2 to \$6.6 trillion (27–29)]. More prosaically, the total required is less than 20% of annual global consumer spending on soft drinks (30).

These results should inform discussions among governments on the magnitude of the financing needs for implementing the CBD Strategic Plan for Biodiversity 2011–2020. A particular challenge will be how to address the current mismatch between the greater resources available in richer countries and the higher potential conservation gains in financially poor, biodiversity-rich countries (31, 32). Resolving the ongoing conservation funding crisis is urgent; it is likely that, the longer that investments in conservation are delayed, the more the costs will grow (23, 33), and the greater will be the difficulty of successfully meeting the targets (6, 34).

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Supplementary Materials

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Pathological α -Synuclein Transmission Initiates Parkinson-like Neurodegeneration in Nontransgenic Mice

Kelvin C. Luk, Victoria Kehm, Jenna Carroll, Bin Zhang, Patrick O'Brien, John Q. Trojanowski, Virginia M.-Y. Lee*

Parkinson's disease is characterized by abundant α -synuclein (α -Syn) neuronal inclusions, known as Lewy bodies and Lewy neurites, and the massive loss of midbrain dopamine neurons. However, a cause-and-effect relationship between Lewy inclusion formation and neurodegeneration remains unclear. Here, we found that in wild-type nontransgenic mice, a single intrastriatal inoculation of synthetic α -Syn fibrils led to the cell-to-cell transmission of pathologic α -Syn and Parkinson's-like Lewy pathology in anatomically interconnected regions. Lewy pathology accumulation resulted in progressive loss of dopamine neurons in the substantia nigra pars compacta, but not in the adjacent ventral tegmental area, and was accompanied by reduced dopamine levels culminating in motor deficits. This recapitulation of a neurodegenerative cascade thus establishes a mechanistic link between transmission of pathologic α -Syn and the cardinal features of Parkinson's disease.

Parkinson's disease (PD) is a multisystem neurodegenerative disorder characterized by two major disease processes: the accumulation of intraneuronal Lewy bodies/Lewy neurites (LBs/LNs) containing misfolded fibrillar α -synuclein (α -Syn), and the selective degeneration of midbrain dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) leading to bradykinesia, tremor, and postural instability (1).

The etiology of these processes remains unclear, although in familial PD, autosomal dominant α -Syn gene mutations or amplifications directly

Department of Pathology and Laboratory Medicine, Institute on Aging and Center for Neurodegenerative Disease Research, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104–4283, USA.

*To whom correspondence should be addressed. E-mail: vmylee@upenn.edu (V.M.Y.L.).

link α -Syn dysfunction to disease causation (2, 3). Moreover, despite the observation that α -Syn pathology is present in virtually all sporadic and familial PD patients (4) and that its distribution correlates with clinical symptoms (5–7), the precise relationship between α -Syn misfolding and DA neuron loss remains controversial because neither transgenic nor neurotoxin-based animal models of PD fully recapitulate the DA neuron degeneration, motor deficits, and the LB/LN-like α -Syn pathology seen in PD (8, 9).

Converging lines of evidence indicate that misfolded fibrillar forms of neurodegenerative disease-related proteins self-propagate and spread between interconnected central nervous system (CNS) regions, suggesting that cell-to-cell transmission of pathological proteins contributes to disease progression (10–15). Indeed, the pattern of LB/LN accumulation in human PD is highly stereotypical, consistent with propagation of disease over functional networks, suggesting that the transmission of pathologic α -Syn and its corruption of

endogenous protein plays a central role in PD pathogenesis and progression (16, 17). Moreover, reports that embryonic mesencephalic neuron grafts in PD patients develop LBs many years after grafting (18, 19) further support the transmissibility of pathologic α -Syn. Consistent with this hypothesis, intracerebral inoculation of α -Syn preformed fibrils (PFFs) composed of recombinant protein accelerates the onset of neurological symptoms and death in transgenic mice overexpressing human A53T mutant α -Syn by enhancing the conversion of endogenous α -Syn into pathological LBs/LNs (20). Although these data implicate α -Syn misfolding and fibrillization as triggering events upstream of neuronal dysfunction/death, minimal DA cell loss was observed, possibly reflecting alterations in the pattern of α -Syn transgene expression and the rapid demise of the inoculated mice.

Because synthetic α -Syn PFFs efficiently seed the aggregation and fibrillization of soluble endogenous α -Syn in primary neuronal cultures

generated from wild-type (WT) mice (21), we asked whether transmission of LB/LN pathology might occur in WT mice in vivo. To answer this, we first determined whether PFFs assembled from recombinant mouse α -Syn initiate pathological α -Syn conversion when introduced into young WT (C57BL/6/C3H) mice by stereotaxic injections targeting the dorsal striatum (fig. S1), a region receptive to PFF uptake (20) and interconnected with multiple CNS nuclei, including midbrain DA neurons. Deposits of hyperphosphorylated α -Syn (pSyn), a marker of human LBs/LNs (22), were visible at the injection site within 30 days of a single unilateral PFF inoculation (Fig. 1, A, B, and G). Intraneuronal α -Syn accumulations, primarily diffuse LN- and LB-like inclusions, were also present in several areas directly interconnected to the striatum that accumulate LBs in human synucleinopathies, most prominently in cortical layers IV and V and the olfactory bulb (Fig. 1, C and D), confirming the pathological conversion

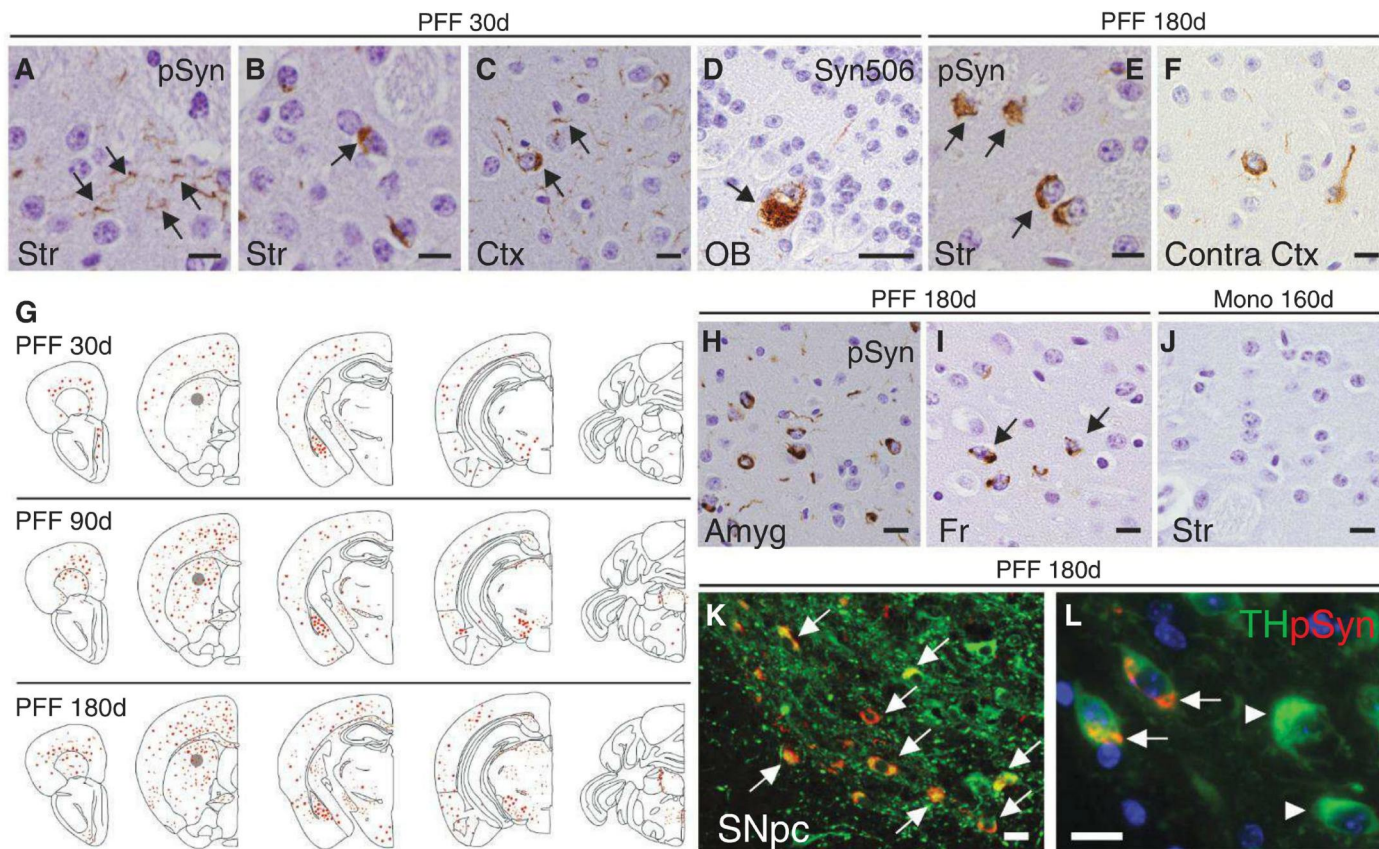


Fig. 1. Intrastriatal inoculation of synthetic mouse α -Syn PFFs seeds the aggregation of endogenous mouse Syn in WT mice. Pathology in brains of C57BL/6/C3H F1 mice after a single unilateral injection of mouse α -Syn PFFs into the dorsal striatum. (A to D) Accumulation of α -Syn in neuritic processes or as pale cytoplasmic inclusions in striatum (Str) and neocortex (Ctx), and olfactory mitral neurons (OB) ipsilateral to the injection at 30 dpi. Black arrows highlight pathology revealed by immunostaining using antibodies to pSyn [(A) to (C)] or Syn506 (D). (E and F) LB-like inclusions in striatum and contralateral neocortex at 180 days postinjection with PFFs. (G) CNS distribution of pSyn accumulations of mice that received a single inoculation of PFFs in the dorsal striatum (indicated by gray circles). Representative maps of

LB/LN-like pathology (red dots and stipples, respectively) in the PFF-injected hemisphere are shown for mice sacrificed at 30, 90, or 180 dpi. (H) α -Syn pathology in amygdala (Amyg) and (I) in frontal cortex (Fr). (J) pSyn staining in ipsilateral striatum 160 dpi with monomeric recombinant α -Syn. (K) Double immunostaining for pSyn (red) and TH (green) in a PFF-injected animal sacrificed at 180 dpi showing LB-like α -Syn pathology in ipsilateral SNpc. (L) High magnification revealing colocalization of pSyn inclusions to DA neurons (white arrows) and reduced TH immunoreactivity compared with unaffected DA neurons (white arrowheads). Images are representative of 3 to 7 mice examined per group (see table S1). Scale bars: 10 μ m [(A) to (C)], [(E) and (F)], and [(H) to (L)]; 25 μ m (D).

of endogenous mouse α -Syn in WT mice. At 30 days postinjection (dpi), pSyn-positive LB-like accumulations were exclusively ipsilateral to the injection site, with the exception of the amygdala, to which the striatum connects bilaterally (23), suggesting that cell-to-cell transmission followed interneuronal connectivity.

LB/LN pathology within affected regions showed markedly increased pSyn immunoreactivity in mice examined 90 and 180 dpi (Fig. 1, E, F, and G). Additional populations, such as the contralateral neocortex, also developed LBs/LNs indicative of progressive spread to CNS regions (Fig. 1, F to I, and table S1). Mapping of pSyn pathology in mice at 30, 90, and 180 dpi (Fig. 1G) revealed a time-dependent dissemination of LBs/LNs between 30 and 180 dpi, with sequential involvement of populations initially unaffected at 30 dpi, including ventral striatum, thalamus, and occipital cortex, along with commissural and brainstem fibers. Although inclusions were absent from multiple regions, including hippocampus, septum, and cerebellum (Fig. 1G and table S1), stereotaxic inoculation of equal quantities of PFFs into the hippocampus resulted in massive accumulation of α -Syn pathology (fig. S2), indicating that neurons in this region

also are susceptible to transmitted pathologic α -Syn. Thus, propagation of LBs/LNs is connectivity-dependent, and this provides an explanation for the nonuniform distribution of LBs/LNs observed in α -Syn PFF-seeded animals. In contrast, monomeric α -Syn or phosphate-buffered saline (PBS) injections did not result in α -Syn pathology at any time point examined (Fig. 1J and fig. S2).

Striatal PFF inoculation led to abundant α -Syn pathology in tyrosine hydroxylase (TH)-positive SNpc DA neurons (Fig. 1, K and L) that project to medium spiny neurons of the dorsal striatum and whose selective degeneration is a major contributor to motor deficits in PD (24). Furthermore, α -Syn pathology in PFF-inoculated mice colocalized with key markers of LBs, including ubiquitin, heat-shock protein 90, and the amyloid-binding dye thioflavin S, indicating that they share common properties with authentic LBs/LNs in human PD (fig. S3).

SNpc α -Syn pathology developed progressively after PFF injection, evolving from pale cytoplasmic accumulations at 30 dpi to dense perinuclear LB-like inclusions by 90 and 180 dpi, particularly among ventromedial SNpc populations (Fig. 2, A to C). Because the nigrostriatal pathway is exclusively unilateral, LB/LN pathol-

ogy in the SNpc was confined to the injected hemisphere at all time points examined (Figs. 1G and 2, A to D). In addition to being absent in ventral tegmental (VTA) neurons, α -Syn pathology was not observed in DA-expressing olfactory glomerular interneurons and arcuate cells of the hypothalamus (fig. S4). Similarly, norepinephrine (locus coeruleus) and serotonergic (Raphe nucleus) populations also remained unaffected at 180 dpi, suggesting that monoaminergic neurons are not equally susceptible to pathological α -Syn spread.

α -Synuclein pathology in the SNpc was accompanied by the gradual and unilateral loss of TH immunoreactivity and neurons (Fig. 2, F to I), suggesting that intraneuronal α -Syn inclusions lead to DA neuron loss. A total of 27.7% of SNpc DA neurons colocalized with pSyn pathology at 30 dpi (Fig. 2K), a stage at which SNpc and VTA DA neuron number were similar in both hemispheres (Fig. 2, L and M). SNpc, but not VTA DA, neurons developed LBs/LNs, confirming transmission of misfolded α -Syn through network projections and suggesting that connectivity is a determinant of susceptibility. The proportion of inclusion-bearing SNpc DA neurons did not increase further but instead declined over

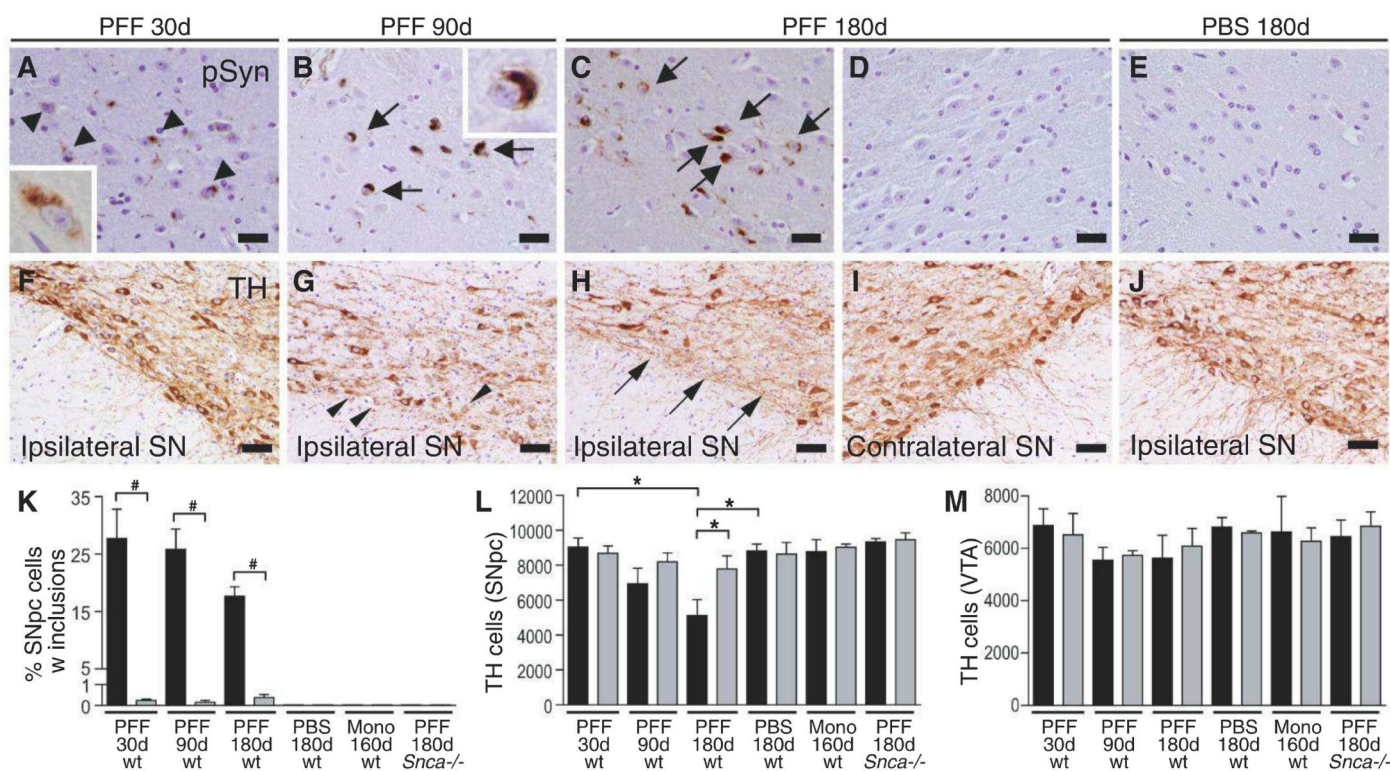


Fig. 2. Seeded α -Syn pathology leads to progressive DA system degeneration. (A to D) pSyn immunostaining in SNpc of mice sacrificed at 30, 90, or 180 days after striatal PFF injection. (A) Diffuse perinuclear pSyn inclusions (black arrowheads) at 30 dpi. (B and C) Dense LB-like inclusions (black arrows) at 90 and 180 dpi. Absence of pSyn pathology in SNpc contralateral to the PFF injection site (D) and in ipsilateral SNpc of PBS-injected mouse (E) at 180 dpi. (F to I) TH immunostaining of SNpc at 30, 90, and 180 days after inoculation with Syn PFFs. Arrowheads in (G) indicate neurons with reduced TH staining in the ipsilateral SNpc at 90 dpi. (H and I) Ventral SNpc, ipsilateral and contralateral

to the site of PFF injection at 180 dpi. Arrows point to areas of DA neuron loss. (J) PBS-injected control at 180 dpi. (K) Percentage of SNpc TH neurons containing pSyn-immunoreactive inclusions for each treatment group. Data for treated ipsilateral (black) and contralateral (gray) hemispheres are shown. $\#P < 0.001$, paired t test ($N = 3$ to 5 mice per group). (L and M) Quantification of TH-immunoreactive neurons in the SNpc and VTA of mice after intrastriatal PFF, monomer or PBS injection. Data represent mean number of cells per region $\pm$ SEM ($N = 3$ to 4 mice per group). $*P < 0.05$, one-way analysis of variance (ANOVA). Scale bars: 25 μ m [(A) to (D)]; 50 μ m [(E) to (J)].

time. There was a concomitant decrease of SNpc DA neurons by 15 and 35% at 90 and 180 dpi, respectively, suggesting that LBs/LNs form before SNpc DA neuron loss. Moreover, DA neurons in the contralateral SNpc and the VTA of both hemispheres (where inclusions were present in <0.2% of neurons) (Fig. 2K) were indistinguishable from those in age-matched PBS- or monomer-injected controls and PFF-injected *Snca*^{-/-} mice, all of which did not develop α -Syn deposits (Fig. 2, D and E, and K to M). Thus, accumulation of pathologic α -Syn appears to be upstream of and directly linked to SNpc DA neuron loss.

Concomitant with the progressive loss of SNpc neurons, striatal concentrations of DA and its metabolites in the injected hemisphere showed progressive reductions after PFF inoculations, whereas contralateral striatal DA levels remain unaltered and noradrenaline (NA) levels were unchanged bilaterally (Fig. 3, A and B, and fig. S5A). Immunoblot analyses confirmed reductions in TH and dopamine transporter (DAT) expression in the ipsilateral striatum (Fig. 3C), consistent with the loss of nigrostriatal DA innervation. Despite the loss of

DA terminals, D1 and D2 dopamine receptor levels remained constant (Fig. 3C and fig. S5C), suggesting the preservation of striatal medium spiny projection neurons.

Given the PD-like Lewy pathology, SNpc neuron loss, and reduced striatal DA levels, PFF-inoculated WT mice might be expected to exhibit altered motor function, as in PD. Although no gross motor or behavioral abnormalities were observed up to 180 dpi, rotarod testing revealed significant and progressive performance deterioration in PFF-injected animals, indicative of impaired motor coordination and balance (Fig. 3D, left). Furthermore, mice with α -Syn pathology performed poorly on the wire-hang test, a complementary measure of motor strength and coordination, declining 53% from baseline at 30 dpi and 81% at 180 dpi (Fig. 3D, middle). However, open-field activity remained unchanged, indicating that overall motor activity was not significantly altered (Fig. 3D, right).

As previously noted, intrastriatal PFF-inoculated mice did not develop hippocampal pathology (Fig. 1G), and no changes in memory function were observed as assessed using the Y maze

(Fig. 3E). This corroborates our interpretation that declining neuronal function is correlated with increasing α -Syn pathology in affected neurons. Moreover, in keeping with the relative sparing of mesolimbic projections, no significant differences were found between PFF-treated and control mice in the tail-suspension test (Fig. 3E, right) a measure of depression-like behavior. The correlation between α -Syn pathology and neuronal deficits was also seen in striatal PFF-inoculated *Snca*^{+/-} mice, where at 180 dpi both SNpc α -Syn pathology and motor impairments were attenuated relative to WT animals (Figs. 3, D and E, and fig. S6A). Finally, striatal inoculation into WT CD1 (fig. S6B) and C57BL/6/SJL mice (table S1) also resulted in transmission of α -Syn pathology, suggesting that cell-to-cell spread of LBs/LNs is independent of mouse genetic background.

In summary, we demonstrate that a single intrastriatal injection of synthetic misfolded α -Syn seeds into WT mice initiates a neurodegenerative cascade characterized by the accumulation of intracellular LB/LN pathology, selective loss of SNpc DA neurons, and impaired

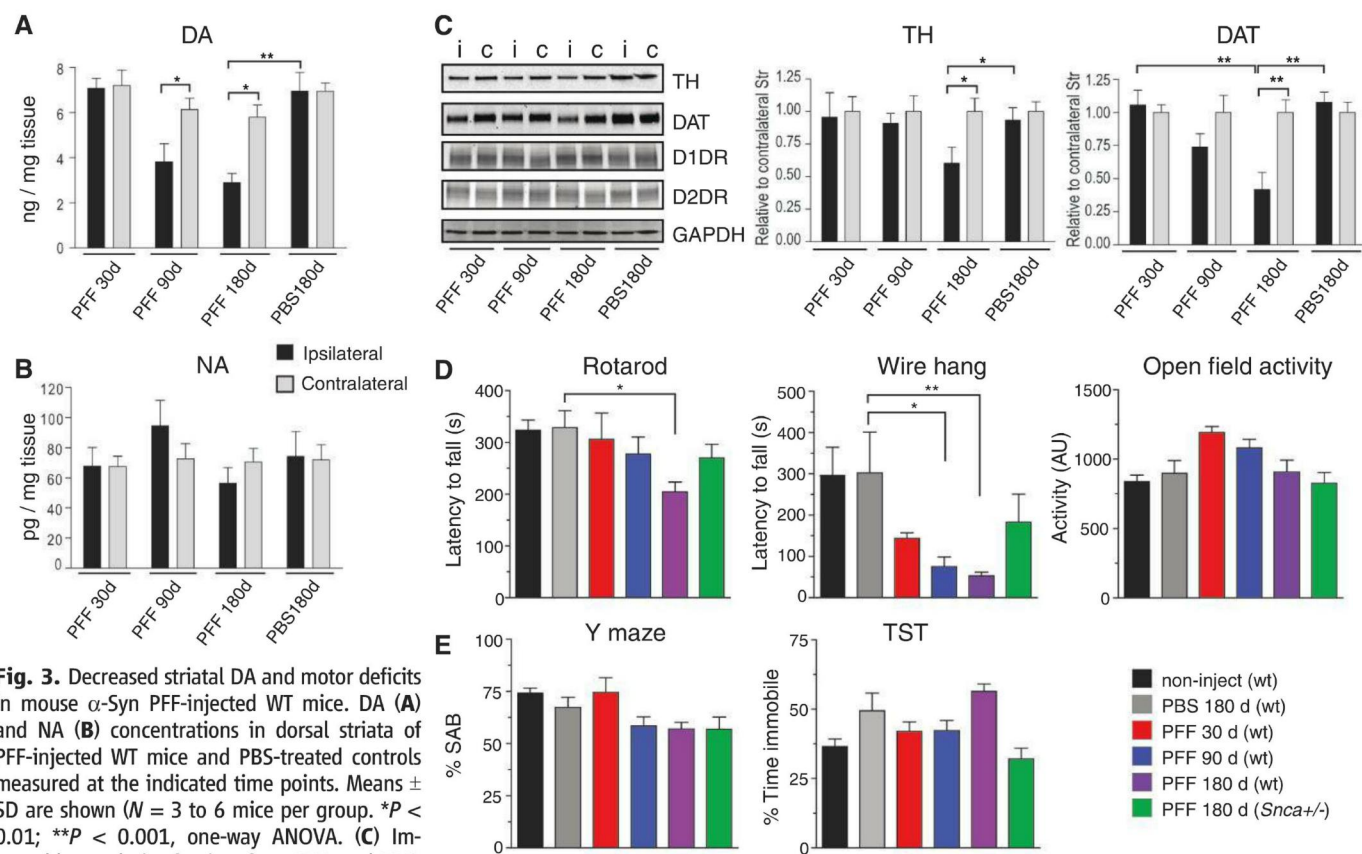


Fig. 3. Decreased striatal DA and motor deficits in mouse α -Syn PFF-injected WT mice. DA (A) and NA (B) concentrations in dorsal striata of PFF-injected WT mice and PBS-treated controls measured at the indicated time points. Means $\pm$ SD are shown ($N = 3$ to 6 mice per group). * $P < 0.01$; ** $P < 0.001$, one-way ANOVA. (C) Immunoblot analysis of striata from PFF- and PBS-treated animals, using antibodies against TH, DAT, D1 dopamine receptor (D1DR), and D2 dopamine receptor (D2DR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control. Mean intensity values are shown for TH and DAT ($N = 3$ to 4 striata per marker). Black and gray bars denote ipsilateral and contralateral regions, respectively. * $P < 0.05$; ** $P < 0.001$, one-way ANOVA. (D and E) Behavioral assessment of WT mice 30, 90, or 180 dpi ($N = 6, 9$, and 17 mice, respectively) after a single unilateral

inoculation of α -Syn PFFs into the striatum. PFF-injected *Snca*^{+/-} mice ($N = 4$), as well as age-matched noninjected ($N = 20$) and PBS-injected ($N = 8$) WT mice are also shown. Results of animals on the rotarod test [(D), left panel] and wire-hang test [(D), middle panel] show progressive deficits in PFF-injected but not control mice. (E) Performance on the Y-maze and tail-suspension test. Data are mean values $\pm$ SD. Differences were established using one-way ANOVA ($P = 0.0012$) with Tukey post-hoc test. * $P < 0.05$; ** $P < 0.01$.

motor coordination. Thus, α -Syn pathology is sufficient to induce the cardinal behavioral and pathological features of sporadic PD.

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Supplementary Materials

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Orbitofrontal Cortex Supports Behavior and Learning Using Inferred But Not Cached Values

Joshua L. Jones,^{1*} Guillem R. Esber,² Michael A. McDannald,² Aaron J. Gruber,³ Alex Hernandez,¹ Aaron Mirenzi,² Geoffrey Schoenbaum^{1,2*}

Computational and learning theory models propose that behavioral control reflects value that is both cached (computed and stored during previous experience) and inferred (estimated on the fly on the basis of knowledge of the causal structure of the environment). The latter is thought to depend on the orbitofrontal cortex. Yet some accounts propose that the orbitofrontal cortex contributes to behavior by signaling “economic” value, regardless of the associative basis of the information. We found that the orbitofrontal cortex is critical for both value-based behavior and learning when value must be inferred but not when a cached value is sufficient. The orbitofrontal cortex is thus fundamental for accessing model-based representations of the environment to compute value rather than for signaling value per se.

Computational and learning theory accounts have converged on the idea that reward-related behavioral control reflects two types of information (1–3). The first is derived from habits, policies, or cached values. These terms reflect underlying associative structures that incorporate a precomputed value stored during previous experience with the relevant cues. Behaviors based on this sort of information are fast and efficient but do not take into account changes in the value of the expected reward. This type of information contrasts with the second category, referred to as goal-directed or model-based, in which the value is inferred from knowledge of the associative structure of the environment, including how to obtain the expected reward, its

unique form and features, and current value. The associative model is stored, but a precomputed value is not. Rather, the value is computed or inferred on the fly when it is needed. Whereas behavior based on inferred value is slower, it can be more adaptive and flexible.

Although evidence suggests that different brain circuits mediate their respective influences (1–3), much of cognitive neuroscience—and particularly neuroeconomics—does not attend to these distinctions. For example, proposals for a common neural currency to allow the comparison of incommensurable stimuli (e.g., apples and oranges) typically do not clearly specify the associative structure underlying the value computation. And because economic value is typically measured through revealed preferences, with no explicit control for the source of the underlying value, it would, by default, include both cached and inferred value, at least as defined by computational and learning theory accounts (1–3).

The calculation of economic value is often assigned to the orbitofrontal cortex (OFC), a prefrontal area heavily implicated in value-guided behavior (4–6). Yet behavioral studies across species implicate this region broadly, not in value-

guided decisions per se, but rather in behaviors that require a new value to be estimated after little or no direct experience (7–14). Further, the OFC is often involved in a behavior that depends on whether learning is required (10, 15, 16), even when that learning does not involve changes in value (17). These data seem to require the OFC to perform one function—anticipating outcomes, in some settings—whereas it performs another, calculating economic value, in others. However, an alternative hypothesis is that the OFC performs the same function in all settings and specifically contributes to value-guided behavior and learning when value must be inferred or derived from model-based representations. We tested this hypothesis in rats using sensory preconditioning and blocking.

In sensory preconditioning, a subject is taught a pairing between two cues (e.g., white noise and tone) and later learns that one of these cues predicts a biologically meaningful outcome (e.g., food) (18). Thereafter, the subject will exhibit a strong conditioned response to both the reward-paired cue and the preconditioned cue. The response to the preconditioned cue differs from the response to the reward-paired cue, in that it cannot be based on a cached value; rather, it must reflect the subject's ability to infer value by virtue of a knowledge of the associative structure of the task (see supplementary discussion for further details). If the OFC is required only for behavior that requires inferred value, then inactivating it at the time of this test should prevent behavior driven by this preconditioned cue, while leaving unimpaired behavior driven by the reward-paired cue.

Cannulae were implanted bilaterally in the OFC of rats [19 controls and 16 inactivated (OFCi)] at coordinates used previously (12, 19) (Fig. 1, A and B). After recovery from surgery, these rats were deprived of food and then trained in a sensory preconditioning task (Fig. 1) (see materials and methods).

In preconditioning, rats were taught to associate two pairs of unrelated auditory cues (A→B and C→D; clicker, white noise, tone, siren; counterbalanced). Food cup responding was measured during presentation of each cue versus baseline

¹Department of Anatomy and Neurobiology, University of Maryland School of Medicine, 20 Penn Street, Baltimore, MD 21201, USA. ²Behavioral Neurophysiology Research Section, Cellular Neurobiology Research Branch, National Institute on Drug Abuse Intramural Research Program, 251 Bayview Boulevard, Baltimore, MD 21201, USA. ³Department of Neuroscience, University of Lethbridge, Lethbridge, Alberta T1K 3M4, Canada.

*To whom correspondence should be addressed. E-mail: geoffrey.schoenbaum@nih.gov (G.S.); josh.jones@nih.gov (J.L.J.)

as an index of conditioning; the rats responded at baseline levels to all cues (Fig. 1, A and B). A two-factor analysis of variance (ANOVA) (cue $\times$ treatment) comparing the percentage of time spent in the food cup during each cue found no effects (F values < 1.27 ; P values > 0.29).

In conditioning, rats were taught that one of the preconditioned cues (B) predicted reward. As a control, the other preconditioned cue (D) was presented without reward. Rats learned to discriminate between the rewarded (B) and nonrewarded (D) cue and to increase responding across sessions during the former more than the latter (Fig. 1, C and D). A three-factor ANOVA (cue $\times$ treatment $\times$ session) revealed significant main effects of cue ($F_{(1,33)} = 170.5$, $P < 0.0001$) and session ($F_{(5,165)} = 54.75$, $P < 0.0001$) and a significant cue $\times$ session interaction ($F_{(5,165)} = 64.6$, $P < 0.0001$) but no significant main effect nor any interactions with treatment (F values < 1.49 , P values > 0.19).

In the probe test, we assessed responding to the preconditioned cues (A and C) after infusions of either saline or a γ -aminobutyric acid agonist cocktail containing baclofen and muscimol. Rats received three presentations of B and D, reinforced as in prior training, followed by six unrewarded presentations of A and C, in a counterbalanced design. Both control and OFCi rats exhibited robust responding to the reward-paired cue (B) (Fig. 1, E and F) and not to the cue that was presented without reward (D). An analysis restricted to the first presentation of each cue, before any reward delivery, revealed a significant main effect of cue ($F_{(1,33)} = 53.21$, $P < 0.0001$) and no significant effect or interaction with treatment (F values < 1.9 , P values > 0.17). However, only controls showed elevated responding to the preconditioned cue (A) that had been paired with the reward-paired cue (B) (Fig. 1E). Controls responded significantly more to this cue than to the preconditioned cue (C), which signals the nonrewarded cue (D), whereas OFCi rats responded to both preconditioned cues similarly and at a level comparable to the responding shown to the cue signaling nonreward (D) (Fig. 1F). A two-factor ANOVA (cue $\times$ treatment) indicated a significant main effect of cue ($F_{(1,33)} = 14.7$, $P < 0.001$) and a significant interaction between cue and treatment ($F_{(1,33)} = 7.33$, $P < 0.01$). Both groups responded significantly more to B than D, and controls responded significantly more to A than to either C or D (Bonferroni post hoc correction; P values < 0.05), whereas OFCi rats responded similarly to these three cues (P values > 0.05).

These results show that the OFC is required when behavior must be based on inferred, but not cached, value. However, they do not address how OFC is involved in learning. To test this question, we used blocking (20). In blocking, a subject is taught that a cue predicts reward (e.g., tone predicts food); later, that same cue is presented together with a new cue (e.g., light-tone), still followed by reward. If this is done, the subject will subsequently show little conditioned responding to

the new cue (e.g., the light in our example). The ability of the original cue to predict the reward is said to block learning. The OFC is not required for this type of blocking (17, 21), which indicates that the OFC is not necessary for modulating learning based on cached value, but this does not address whether the OFC is necessary for blocking on the basis of inferred value. If the OFC performs the same function during learning, then inactivating the OFC during blocking with the preconditioned cue should result in unblocking.

To test this, we trained a subset of rats from the experiment described above in an inferred value blocking task (Fig. 2) (see supplementary materials). Rats underwent 2 days of training in which the preconditioned auditory cues (A and C) were presented with novel light cues (X and Y; house light, flashing cue light; counterbalanced). Both pairs of cues were reinforced with the same reward previously paired with B (AX $\rightarrow$ sucrose; CY $\rightarrow$ sucrose). Before each session, rats received infusions of saline or the baclofen-muscimol cock-

tail. Both groups showed a significant increase in responding, and there was no overall difference between the two groups (Fig. 2A). A three-factor ANOVA (treatment $\times$ cue $\times$ session) demonstrated a significant effect of session ($F_{(1,19)} = 16.53$, $P < 0.001$), but there was no significant main effect nor any interactions with treatment (F values < 1.44 , P values > 0.24).

One day after blocking, these rats were presented with several AX and CY reminder trials, followed by unreinforced trials in which cues X and Y were presented. Controls exhibited significantly higher conditioned responding to the control cue (Y) than to the blocked cue (X). Indeed, responding to the blocked cue was no greater than baseline (Fig. 2B) ($t_{(1,11)} = 0.67$, $P = 0.52$). By contrast, OFCi rats showed increased responding to both cues, consistent with an inability to use inferred value to block learning (Fig. 2B). A two-factor ANOVA (treatment $\times$ cue) revealed a significant interaction between cue and treatment ($F_{(1,19)} = 7.70$, $P = 0.012$), and controls responded significantly more

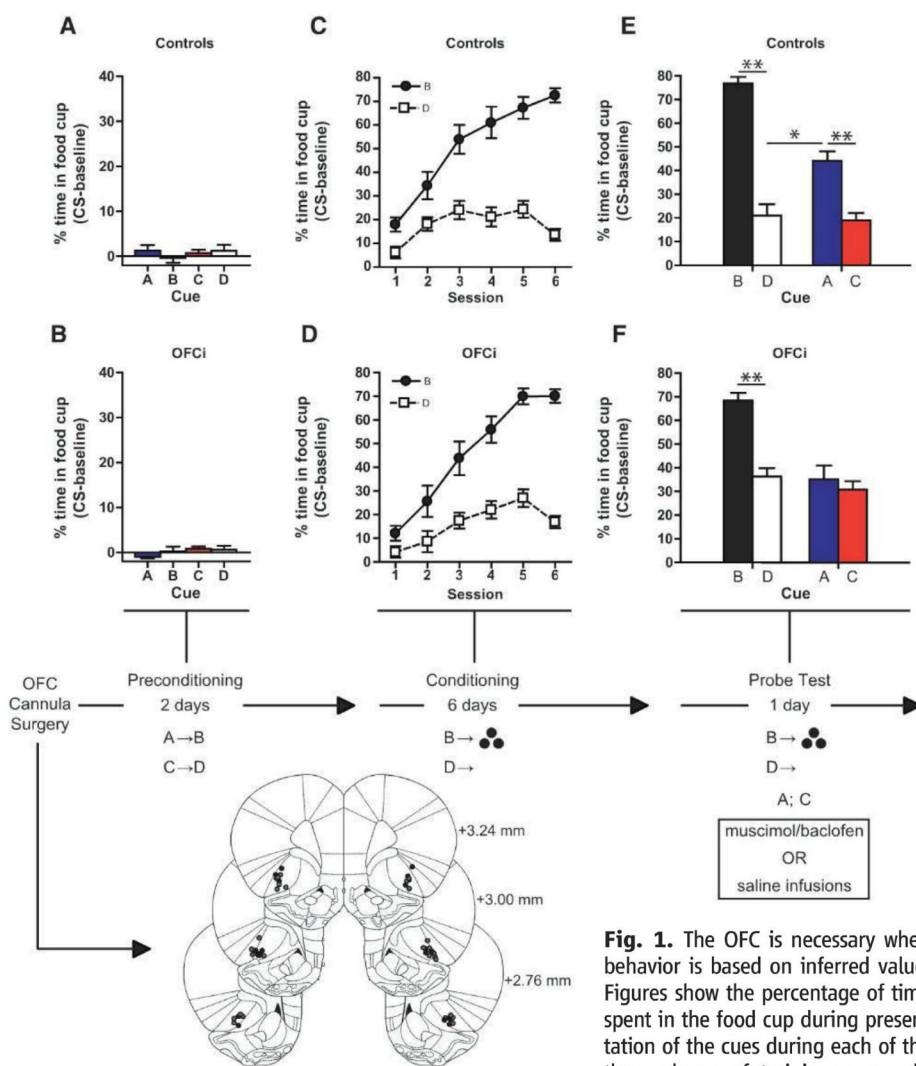


Fig. 1. The OFC is necessary when behavior is based on inferred value. Figures show the percentage of time spent in the food cup during presentation of the cues during each of the three phases of training: preconditioning (A and B), conditioning (C and D), and the probe test (E and F). OFC was inactivated only during the probe test. Cannulae positions are shown below; vehicle (black circles), OFCi (gray circles). * $P < 0.05$, ** $P < 0.01$ or better. Error bars show SEM.

to Y than to X (Bonferroni post hoc correction; $P < 0.05$), whereas OFCi rats responded similarly to these two cues ($P > 0.05$).

These findings demonstrate that the OFC is involved in value-based behavior when the value must be inferred from an associative model of the task but not when the same behavior can be based on a value cached or stored in cues during past experience. This is consistent with previous results implicating the OFC in changes in conditioned responding after reinforcer devaluation (7, 8, 10, 13, 22, 23). Our results confirm that OFC is required for knowledge of the associative structure at the time of the decision, rather than the results owing to some idiosyncratic involvement in taste perception, reward learning per se, or devaluation. This is because, in our task, we did not alter the reward in any way. By inactivating only at the time of the probe test, we show clearly that the OFC is required for using the previously acquired associative structure. Thus, the structure may be stored elsewhere, but it cannot be applied to guide behavior effectively without the OFC. By including an explicit control for cached value, we show that this deficit is specific for inferred value at the time of decision-making. These data are also consistent with several functional magnetic resonance imaging studies showing that neural activity in OFC may be particularly well-tuned to reflect model-based information at the time of decision-making (13, 24). In this regard, it is notable that this same function was also required for modulating learning, because the pre-

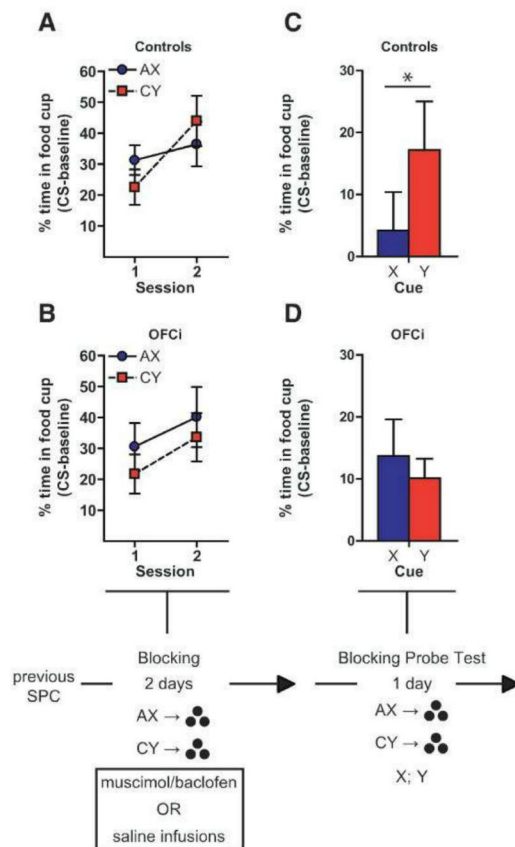
conditioned cue also failed to function as a blocker after OFC inactivation. This suggests that the OFC plays a general role in signaling inferred value, which might be used by other brain regions for a variety of purposes, rather than having a special role in the service of decision-making.

It is remarkable that the inferred value signal evoked by this cue resulted in blocking. Blocking normally uses a cue that has been paired directly with reward. Theoretical accounts focus on the fact that the value of the expected reward is already fully predicted, and therefore, there is no prediction error to drive learning (25–27). However, these accounts do not specify the source of this value, and generally, it is assumed to be a sort of cached or general value. In fact, temporal-difference reinforcement learning is specific on this point (3). Our experiment shows that inferred value can also modulate learning by serving as a blocking cue, which allows learning to be modulated, not only by experienced information, but also by inferred knowledge.

These results also contradict the argument that the OFC is specifically tasked with calculating value in a common currency, devoid of identifying information about identity and source, because the OFC was necessary for value-based behavior only when calculation of that value required a model-based representation of the task. Indeed, the OFC is necessary for behavior and learning when only the identity of the reward is at issue (17, 28), which suggests that the OFC functions as part of a circuit that maintains and uses the

states and transition functions that make up model-based control systems, rather than as an area that simply calculates general or common value. Indeed, none of these results require that value be calculated within OFC at all. Although radical, such speculation is in line with evidence that other prefrontal regions do as well, or better, than OFC in representing general outcome value (29). Moreover, whereas activity in some OFC neurons correlates with economic value, representations are usually much more specific to elements of task structure (29, 30). The OFC may only be necessary for economic decision-making insofar as the value required reflects inferences or judgments analogous to what we have tested here. Data implicating the OFC in the expression of transitive inference (11) or willingness to pay (14) may reflect such a function, because, in each setting, the revealed preferences are expressed after little or no experience with the imagined outcomes. Limited experience, a defining feature of economic decision-making (5), would minimize the contribution of cached values and so bias subjects to rely on model-based information for the values underlying their choices.

Fig. 2. The OFC is necessary when learning is based on inferred value. Figures show the percentage of time spent in the food cup during presentation of the cues during blocking (A and B) and the subsequent probe test (C and D). OFC was inactivated during blocking. * $P < 0.05$, ** $P < 0.01$ or better. Error bars show SEM.



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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 and S2
References (31–36)

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Akt-Mediated Regulation of Autophagy and Tumorigenesis Through Beclin 1 Phosphorylation

Richard C. Wang,^{1,2} Yongjie Wei,^{2,3,4} Zhenyi An,^{2,3} Zhongju Zou,^{2,3,4} Guanghua Xiao,⁵ Govind Bhagat,⁶ Michael White,⁷ Julia Reichelt,⁸ Beth Levine^{2,3,4,9*}

Aberrant signaling through the class I phosphatidylinositol 3-kinase (PI3K)–Akt axis is frequent in human cancer. Here, we show that Beclin 1, an essential autophagy and tumor suppressor protein, is a target of the protein kinase Akt. Expression of a Beclin 1 mutant resistant to Akt-mediated phosphorylation increased autophagy, reduced anchorage-independent growth, and inhibited Akt-driven tumorigenesis. Akt-mediated phosphorylation of Beclin 1 enhanced its interactions with 14-3-3 and vimentin intermediate filament proteins, and vimentin depletion increased autophagy and inhibited Akt-driven transformation. Thus, Akt-mediated phosphorylation of Beclin 1 functions in autophagy inhibition, oncogenesis, and the formation of an autophagy-inhibitory Beclin 1/14-3-3/vimentin intermediate filament complex. These findings have broad implications for understanding the role of Akt signaling and intermediate filament proteins in autophagy and cancer.

Mutations leading to activation of the serine/threonine kinase Akt are frequent in human cancer (1). Akt has many downstream targets involved in tumorigenesis, including mTOR (mammalian target of rapamycin) (2). Akt also inhibits autophagy (3), a lysosomal degradation pathway that removes unwanted or damaged cellular constituents and functions in tumor suppression (4). Akt suppression of autophagy can be mediated by activation of mTOR, which inhibits the autophagy-initiating Unc-51–like kinase 1 (ULK1) kinase complex (4).

We investigated whether Akt inhibits autophagy by directly regulating the core autophagy machinery independently of mTOR. Expression of

constitutively active myristoylated (5) and tagged Akt1 (Flag-tagged myr-Akt) in HeLa cells inhibited autophagy during growth in normal medium; in response to serum and amino acid starvation (a physiological inducer of autophagy); in response to treatment with an adenosine 5'-triphosphate (ATP)-competitive inhibitor of mTOR, Torin1 (6); and in response to both starvation and Torin1 treatment (Fig. 1, A and B). In all conditions, cells expressing myr-Akt1 had decreased numbers of puncta upon transfection with a fusion protein of green fluorescent protein with LC3 (GFP-LC3), a fluorescent marker of autophagosomes; increased amounts of p62 (a substrate that is degraded by autophagy); and increased amounts of the cytosolic nonlipidated form of LC3 (LC3-I) and of total LC3 (7). Amounts of phospho-4E-BP1, a phosphorylation target of mTOR, were decreased in Torin1-treated cells, including those expressing myr-Akt1. Thus, myr-Akt1 suppresses basal autophagy, starvation-induced autophagy, and Torin1-induced autophagy, indicating that active Akt can inhibit autophagy through mTOR-independent mechanisms.

We examined whether autophagy execution proteins could be targets of Akt. We focused on Beclin 1 because of its role in autophagy and tumor suppression (4). Endogenous Akt coimmunoprecipitated with endogenous Beclin 1 in HeLa cells, and this interaction was weakened by starvation (Fig. 1C). In contrast, the interaction of

myr-Akt1 with a Flag epitope-tagged construct of Beclin 1 was not affected by starvation (fig. S1). Kinase prediction algorithms (8, 9) showed Beclin 1 to contain a motif (R-X-X-R-X-X-S295) that resembles the consensus Akt phosphorylation motif (R-X-R-X-X-S/T) (10) and another sequence (R-X-X-S234) that corresponds to a 14-3-3 protein binding motif (which may be generated by Akt phosphorylation) (fig. S2A) (11). Phospho-specific antibodies against these two candidate phosphorylation sites in Beclin 1 (S234 and S295) recognized wild-type Flag-Beclin 1 expressed in HeLa cells, and immunoreactivity was decreased with the corresponding Flag-Beclin 1 alanine substitution mutant (fig. S2B). Glutathione S-transferase (GST)–Akt1 phosphorylated Beclin 1 S295 but not Beclin 1 S234 in vitro (fig. S2C), and this was partially blocked by treatment with two Akt inhibitors, MK-2206 and Akt inhibitor X (Fig. 1D). Expression of active Akt1 (myr-Akt1) increased and expression of a catalytically inactive, non-phosphorylatable Akt1 mutant (K179M/T308A/S473A; DN-Akt1) decreased, respectively, phosphorylation of Flag-Beclin 1 S295 and Flag-Beclin 1 S234 in HeLa cells (fig. S2D). Expression of myr-Akt1 also led to phosphorylation of endogenous Beclin 1 S295 and endogenous Beclin 1 S234, which was not reversed by mTOR inactivation with Torin1 (Fig. 1E). Endogenous Beclin 1 S295 phosphorylation increased when starved HeLa cells were fed with normal medium (fig. S2E). Together, these studies demonstrate that Beclin 1 is phosphorylated by Akt on residue 295 (and possibly 234) in an mTOR-independent manner.

We compared the phosphorylation of Beclin 1 S295 in three paired sets of tumor cell lines with and without Akt activation (Fig. 1F). Melanoma cells with mutant phosphatase and tensin (PTEN) (WM793) had more phosphorylation of Beclin 1 S295 than did those with wild-type PTEN (451Lu) (12). U87-MG glioblastoma cells with high Akt activity due to inactivating mutations in PTEN showed more phosphorylation of Beclin 1 S295 than did U87-MG cells in which wild-type PTEN was reintroduced (13). In breast carcinoma cells, S295 phosphorylation was detected in MCF10A-DCIS cells with an activating H1047R mutation in *PIK3CA* but not in MDA-MB231 cells lacking constitutive Akt activation (14). Thus, in three different tumor types activation of Akt is associated with phosphorylation of Beclin 1 S295, indicating that phosphorylation of Beclin 1 S295 may be common in human tumors with activated Akt.

¹Department of Dermatology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. ²Center for Autophagy Research, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. ³Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. ⁴Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. ⁵Department of Clinical Sciences, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. ⁶Department of Pathology and Cell Biology, Columbia University Medical Center and New York Presbyterian Hospital, New York, NY 10032, USA. ⁷Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. ⁸Institute of Cellular Medicine, University of Newcastle, Framlington Place, NE2 4HH Newcastle upon Tyne, UK. ⁹Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA.

*To whom correspondence should be addressed. E-mail: beth.levine@utsouthwestern.edu

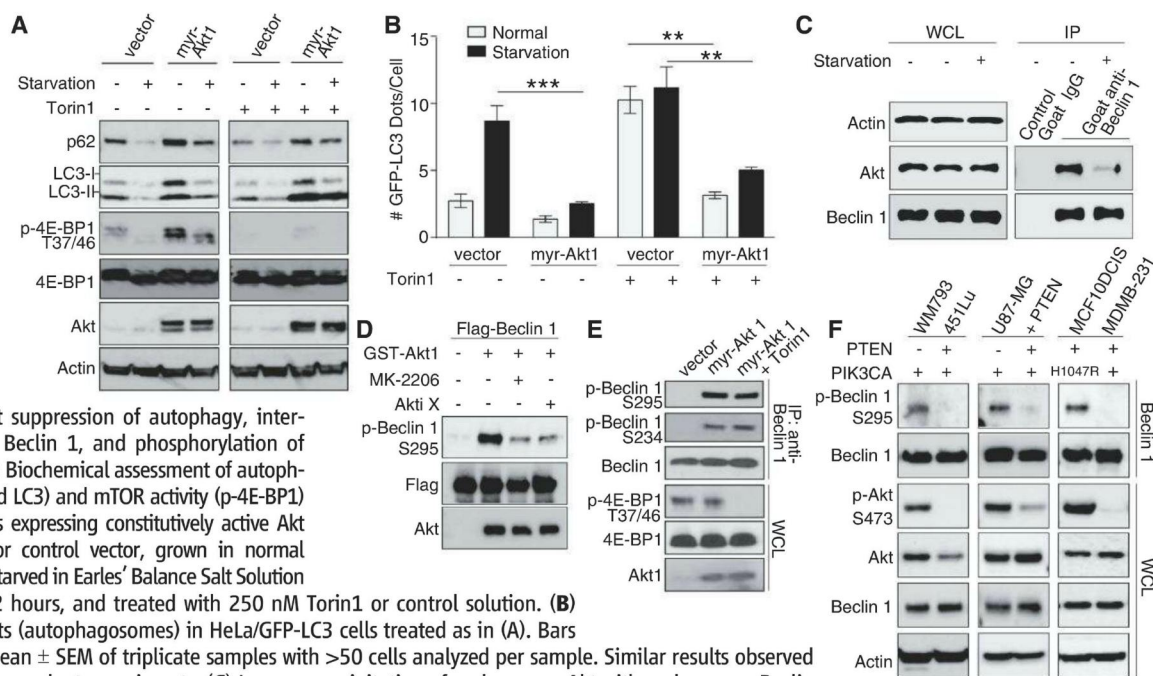
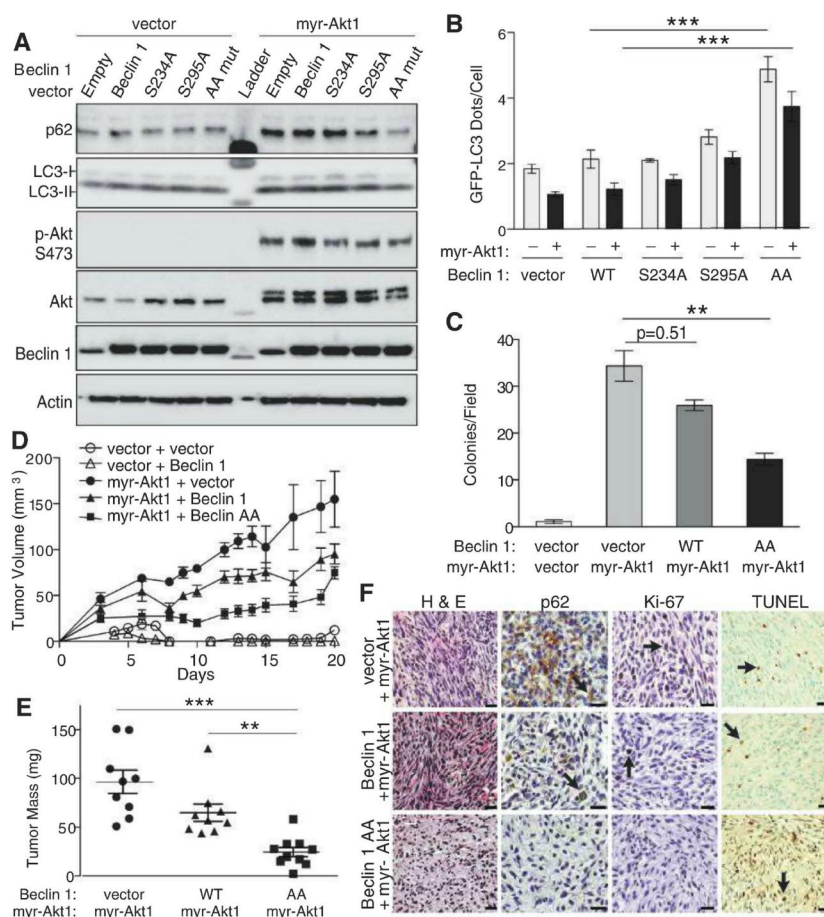


Fig. 2. Effect of a nonphosphorylatable mutant of Beclin 1 [Beclin 1 S234A/S295A (AA)] on autophagy, anchorage-independent growth, and Akt-mediated tumorigenesis. (A) Biochemical assessment of autophagy in Rat2 fibroblasts expressing indicated Flag-Beclin 1 and Akt constructs. (B) GFP-LC3 dots in Rat2 cells transduced with indicated lentiviral vectors and transfected with GFP-LC3. Bars represent mean $\pm$ SEM of triplicate samples with >50 cells analyzed per sample. Similar results observed in three independent experiments. (C) Soft agar colonies formed by Rat2 fibroblasts transduced with indicated vectors. Bars represent mean $\pm$ SEM of triplicate samples of ≥ 12 random images analyzed by using ImageJ. Similar results observed in three independent experiments. (D) Xenograft tumor volumes formed by Rat2 fibroblasts transduced with indicated vectors (P < 0.001 for myr-Akt1 + Beclin 1 AA group versus myr-Akt1 + vector or myr-Akt1 + Beclin 1 groups; linear-mixed effect model). (E) Tumor weights at day 21. (F) Representative images of tumors formed by Rat2 fibroblasts. Arrows denote representative p62-, Ki-67-, and TUNEL-positive cells. Scale bars, 20 μ m. For (D) and (E), results represent mean $\pm$ SEM for all tumors in each genotype (9 or 10 per group). * P < 0.05, ** P < 0.01, *** P < 0.001; Tukey test.



We transfected MCF7 human breast carcinoma cells [which express low amounts of endogenous Beclin 1 (15)] with GFP-LC3 and wild-type Beclin 1 or Beclin 1 S295A or AA mutants (fig. S3A). Cells transfected with S295A and AA mutants had increased basal (but not starvation-induced) autophagy (fig. S3, B and C). Inhibition of Akt by MK-2206 (fig. S3D) increased basal autophagy in MCF7 cells to a lesser extent in cells transfected with Beclin 1 AA than in cells transfected with wild-type Beclin 1 (fig. S3E). Conversely, expression of active Akt decreased basal autophagy in all MCF7 cells, but cells expressing Beclin 1 AA showed more autophagy than did cells expressing wild-type Beclin 1 or vector alone (fig. S3, F and G). Thus, Akt appears to inhibit basal autophagy both through Beclin 1 phosphorylation-dependent and -independent mechanisms.

To examine the role of Akt-mediated Beclin 1 phosphorylation in Akt-driven tumorigenesis, we transduced rat fibroblasts with myr-Akt1 [which transforms rat fibroblasts (16)] and either wild-type Beclin 1 or Beclin 1 phosphorylation site mutants. Myr-Akt1 suppressed autophagy in Rat2 fibroblasts (Fig. 2, A and B), reduced coimmunoprecipitation of class III phosphatidylinositol 3-kinase (PI3K) Vps34 with Beclin 1 (fig. S4A), and decreased Beclin 1-associated lipid kinase activity (fig. S4B). The autophagy suppressive effects of active Akt were largely prevented by expression of the Beclin 1 AA mutant and mildly decreased by the Beclin 1 S295A mutant (Fig. 2, A and B). Expression of myr-Akt1 had minimal effects on the interaction of Beclin 1 AA and Vps34 or on the amounts of Beclin 1 AA-associated Vps34 activity (fig. S4, A and B). Thus, myr-Akt1 suppresses Beclin 1-associated Vps34 activity and autophagy in a manner that is partially reversed by a Beclin 1 mutant resistant to Akt-mediated phosphorylation.

In an anchorage-independence growth assay, short hairpin RNA (shRNA) depletion of Beclin 1 (fig. S5) or myr-Akt1 expression caused Rat2 fibroblasts to form numerous colonies in soft agar (Fig. 2C). Both the number and size of colonies formed by myr-Akt1-expressing cells were reduced by coexpression of Beclin 1 AA (Fig. 2C and fig. S6, A and B). Rat2 cells expressing myr-Akt1 also had higher amounts of endogenous Beclin 1 S295 phosphorylation than that of control cells (Fig. 3B). Thus, active Akt1 promotes Beclin 1 S295 phosphorylation, and expression of a nonphosphorylatable mutant of Beclin 1 suppresses Akt-mediated transformation in vitro. Inactivation of Beclin 1 by Akt-mediated phosphorylation may therefore contribute to Akt's transforming properties.

Beclin 1 AA also suppressed myr-Akt1-driven tumorigenesis in vivo. All myr-Akt1-expressing Rat2 cells formed tumors in immunodeficient non-obese diabetic (NOD) severe combined immunodeficient (SCID) mice, but tumor growth rate and tumor mass upon necropsy were less for cells expressing Beclin 1 AA than for cells expressing wild-type Beclin 1 or myr-Akt1 alone (Fig. 2, D and E, and fig. S6C). Tumors expressing myr-Akt1 alone had numerous cells displaying p62 immunore-

activity (indicating autophagy suppression), whereas very few p62 immunoreactive cells were detected in tumors from cells expressing both myr-Akt1 and Beclin 1 AA (Fig. 2F). Tumors expressing myr-Akt1 alone or active Akt and Beclin 1 resembled fibrosarcomas (Fig. 2F and table S1); 94% (17 of 18) showed tissue invasion, and 50% (9 of 18) exhibited nuclear pleomorphism and decreased nucleus:cytoplasmic ratios. Tumors expressing myr-Akt1 and Beclin 1 AA formed less cellular, more disorganized tumors with limited or no tissue invasion; 60% (6 of 10) displayed degenerative features with numerous pyknotic nuclei. Tumors expressing active Akt alone or with Beclin 1 had higher mean mitotic counts ($n = 8.39$ and 8.37 mean number of mitotic figures per high power field, respectively) than those of tumors expressing myr-Akt1 and Beclin 1 AA ($n = 3.67$ mean number of mitotic figures per high power field) ($P < 0.0001$). They also had increased Ki-67 labeling (fig. S6D) and decreased terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining (fig. S6E). Thus, the expression of a mutant of Beclin 1 that cannot be phosphorylated by Akt increases tumor

cell autophagy, decreases tumor growth rate and size, decreases tumor cellular proliferation, and increases tumor cell death. These results suggest a role for Akt-mediated Beclin 1 phosphorylation in the tumorigenic effects of Akt.

To investigate how Akt phosphorylation of Beclin 1 inhibits autophagy, we determined whether Akt-mediated phosphorylation of Beclin 1 generates a 14-3-3 binding motif (10). Endogenous 14-3-3 proteins and Beclin 1 coimmunoprecipitated in HeLa cells, and this interaction decreased during starvation (Fig. 3A). Mutation of predicted Beclin 1/14-3-3 binding sites, S234A or S295A, weakened the Beclin 1/14-3-3 interaction, and the double mutation (AA) nearly completely abolished Beclin 1/14-3-3 binding (fig. S7A). Similar amounts of Atg14—a component of the autophagy-inducing Beclin 1/Class III PI3K complex (17)—immunoprecipitated with wild-type and nonphosphorylatable mutants of Beclin 1, indicating that these mutations do not cause major alterations in protein stability or folding. Expression of myr-Akt1 blocked starvation-induced disruption of 14-3-3/Beclin 1 binding, and conversely, expression of DN-Akt1 inhibited 14-3-3/Beclin 1 binding during growth

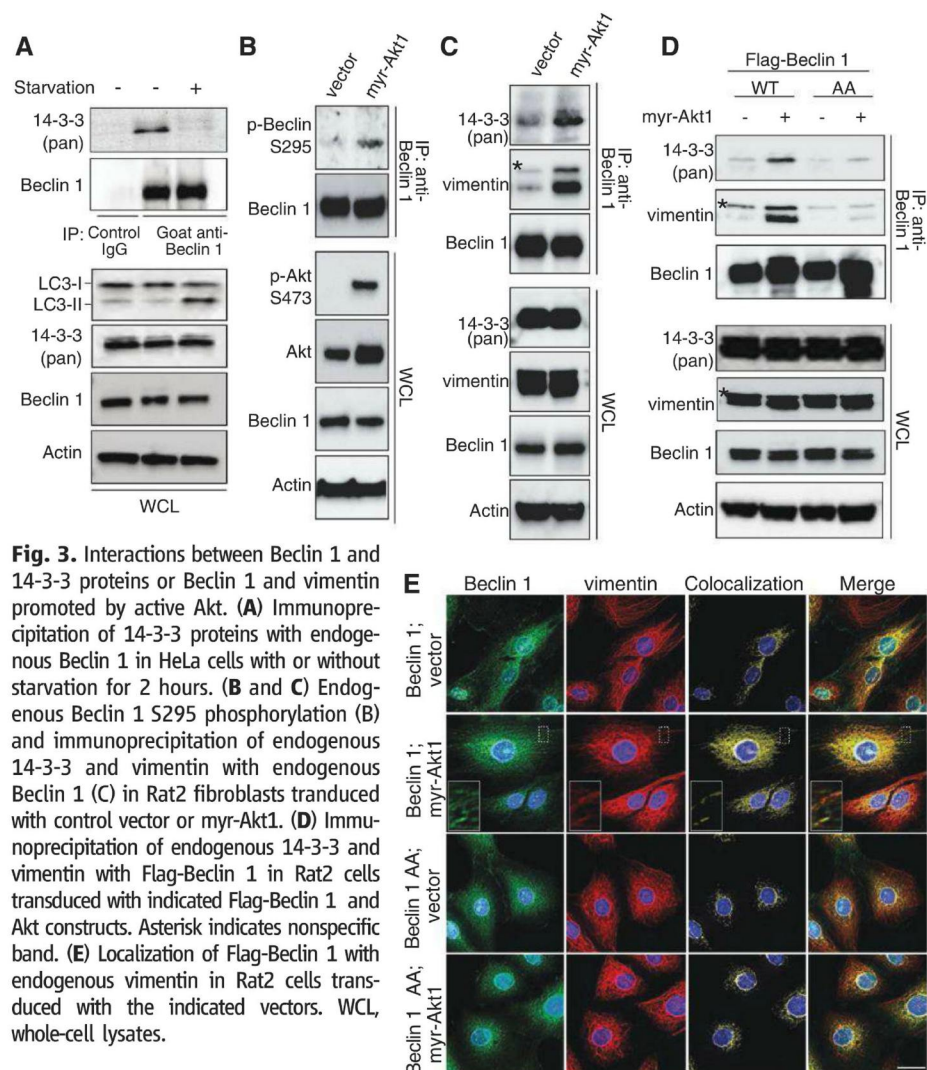


Fig. 3. Interactions between Beclin 1 and 14-3-3 proteins or Beclin 1 and vimentin promoted by active Akt. (A) Immunoprecipitation of 14-3-3 proteins with endogenous Beclin 1 in HeLa cells with or without starvation for 2 hours. (B and C) Endogenous Beclin 1 S295 phosphorylation (B) and immunoprecipitation of endogenous 14-3-3 and vimentin with endogenous Beclin 1 (C) in Rat2 fibroblasts transduced with control vector or myr-Akt1. (D) Immunoprecipitation of endogenous 14-3-3 and vimentin with Flag-Beclin 1 in Rat2 cells transduced with indicated Flag-Beclin 1 and Akt constructs. Asterisk indicates nonspecific band. (E) Localization of Flag-Beclin 1 with endogenous vimentin in Rat2 cells transduced with the indicated vectors. WCL, whole-cell lysates.

in normal medium (fig. S7B). Thus, Beclin 1 interacts with 14-3-3 proteins through S234 and S295, and this interaction is negatively regulated by starvation and Akt inhibition.

14-3-3 proteins can regulate their binding partners' functions through interactions with intermediate filaments (18, 19). The intermediate filaments, keratin 18 (K18) and vimentin, immunoprecipitated with Flag-Beclin 1 in HeLa cells, and this was reduced by expression of DN-Akt1 (fig. S7C). Immunoprecipitation of Flag-Beclin 1 was increased with mutants of vimentin and K18 with increased binding to 14-3-3 proteins [K18 R89C (20)] but decreased with mutants with decreased binding to 14-3-3 proteins [K18S33A (20) and vimentin S39A] (fig. S7, D and E). The Flag-Beclin 1 AA mutant did not immunoprecipitate with wild-type vimentin-GFP (fig. S7F). Small interfering RNA targeted against 14-3-3 ϵ abolished the interaction between Flag-Beclin 1 and vimentin-GFP (fig. S7G). Thus, Beclin 1 interacts with 14-3-3 proteins and intermediate filament proteins through a mechanism involving the S234 and S295 Akt phosphorylation/14-3-3 binding sites of Beclin 1 and the 14-3-3 binding sites of intermediate filament proteins.

In Rat2 cells, expression of myr-Akt1 increased the interactions of endogenous 14-3-3 proteins and vimentin [the major intermediate filament protein expressed in fibroblasts (21)] with endogenous Beclin 1 in parallel with its increased phosphoryl-

ation (Fig. 3, B and C). Expression of active Akt had little effect on the binding of Beclin 1 AA with 14-3-3 proteins and vimentin (Fig. 3D). Thus, active Akt may promote the interaction of Beclin 1 with vimentin through phosphorylation of Beclin 1 and generation of 14-3-3 binding sites.

Wild-type Beclin 1 had a diffuse cytoplasmic localization in the absence of active Akt expression, and localization of Beclin 1 with vimentin was observed primarily in a perinuclear pattern (Fig. 3E). Expression of myr-Akt1 redistributed wild-type Beclin 1 into a reticular pattern and increased Beclin 1/vimentin colocalization but did not have these effects on Beclin 1 AA. Conversely, starvation decreased Beclin 1/vimentin colocalization in a reticular pattern (fig. S7H). Thus, active Akt enhances the colocalization of Beclin 1 with vimentin in Rat2 cells in a manner that requires the Beclin 1 Akt phosphorylation and 14-3-3 binding sites S234 and S295.

In Rat2 cells, two different shRNAs that target vimentin (fig. S8A) increased autophagy (Fig. 4A). Vimentin appears to inhibit autophagy downstream of Beclin 1 phosphorylation because depletion of vimentin also increased autophagy in Rat2 fibroblasts expressing myr-Akt1 (Fig. 4B and fig. S8B). This increased autophagy was associated with inhibition of Akt-mediated transformation; vimentin shRNAs inhibited the number and size of Rat2 colonies formed in soft agar (Fig. 4C and fig. S8C).

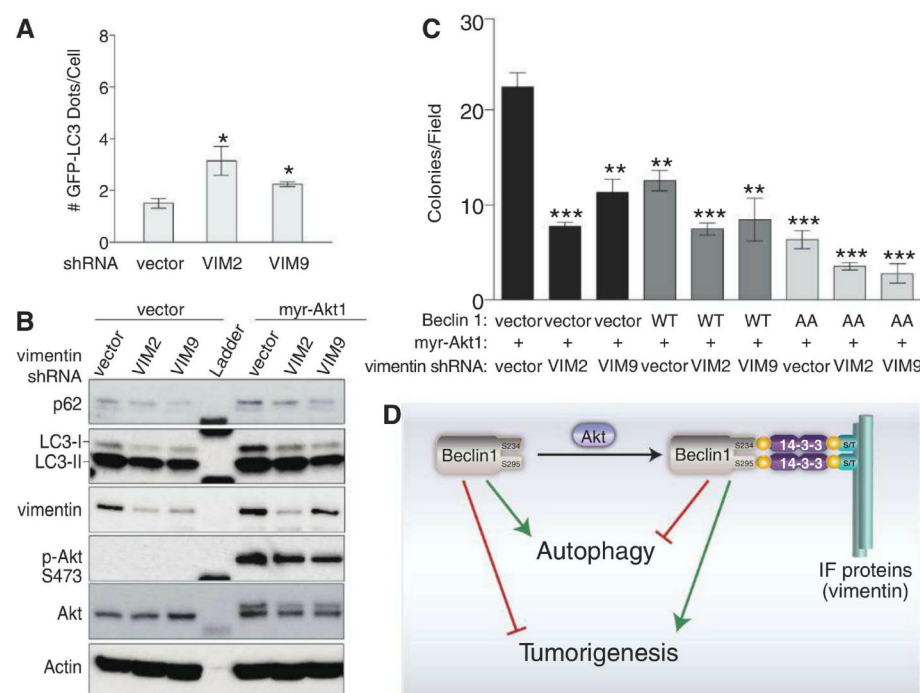


Fig. 4. Effects of vimentin on autophagy and Akt-mediated transformation. (A) GFP-LC3 dots in Rat2 cells transduced with indicated vimentin shRNA and transfected with GFP-LC3. Bars represent mean $\pm$ SEM of triplicate samples with >50 cells analyzed per sample. Similar results observed in three independent experiments. (B) Biochemical assessment of autophagy in Rat2 cells transduced with indicated vimentin shRNA and myr-Akt1 or control vector. (C) Soft agar colonies formed by Rat2 fibroblasts transduced with indicated vectors. Bars represent mean $\pm$ SEM of triplicate samples of ≥ 12 random images analyzed by using ImageJ. (D) Speculative model for relations between Akt phosphorylation of Beclin 1; formation of a complex with Beclin 1, 14-3-3 proteins, and intermediate filament (IF) proteins; autophagy; and tumorigenesis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Student's t test. In (A) and (C), all comparisons are with the first bar in graph.

Thus, the regulation of Beclin 1/vimentin interactions by active Akt may be a mechanism for both inhibition of autophagy by intermediate filaments and for Akt-mediated transformation. As in the case of the interaction of autophagy/Beclin-1 regulator 1 (AMBRA1) with the dynein motor complex (22), these data indicate that interactions between core autophagy proteins and cytoskeletal elements may regulate autophagy.

Our findings demonstrate a link between oncogenic signaling, the core autophagy machinery, and cytoskeletal proteins in the intermediate filament family. Akt signaling, intermediate filaments, and 14-3-3 proteins may be mechanistically linked to autophagy inhibition and tumorigenesis through regulation of the Beclin 1 complex (Fig. 4D). These findings also demonstrate a specific mechanism by which autophagy may be suppressed in human cancer. Cross-talk between oncogenic kinases and autophagy proteins might represent a fundamental mechanism underlying the regulation of mammalian cell growth control and cancer.

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11. In the mutants, other amino acids were substituted at certain locations; for example, K179M indicates that lysine at position 179 was replaced by methionine. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1225967/DC1
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A Rab32-Dependent Pathway Contributes to *Salmonella* Typhi Host Restriction

Stefania Spanò and Jorge E. Galán*

Unlike other *Salmonellae*, the intracellular bacterial human pathogen *Salmonella* Typhi exhibits strict host specificity. The molecular bases for this restriction are unknown. Here we found that the expression of a single type III secretion system effector protein from broad-host *Salmonella* Typhimurium allowed *Salmonella* Typhi to survive and replicate within macrophages and tissues from mice, a nonpermissive host. This effector proteolytically targeted Rab32, which controls traffic to lysosome-related organelles in conjunction with components of the biogenesis of lysosome-related organelle complexes (BLOCs). RNA interference-mediated depletion of Rab32 or of an essential component of a BLOC complex was sufficient to allow *S. Typhi* to survive within mouse macrophages. Furthermore, *S. Typhi* was able to survive in macrophages from mice defective in BLOC components.

The bacterial pathogen *Salmonella enterica* comprises thousands of serovars (i.e., variants that can be distinguished by their surface antigen composition) that as a whole can infect a large number of vertebrate species (1, 2). Some serovars can infect a broad range of hosts, whereas others are extremely host specific. For example, *Salmonella enterica* serovar Typhi (*S. Typhi*) can only infect humans, although experimentally it can also infect higher primates (3). *S. Typhi* causes typhoid fever, a life-threatening systemic disease that every year kills 200,000 people worldwide (4–6). Although the genome

sequences of several host-specific and broad-host *Salmonellae* are available, the molecular bases for this host adaptation are unknown (7, 8). It is believed that genome reduction most likely played a central role in the narrowing of *S. Typhi*'s host range (9). This host restriction is also manifested at the cellular level because, unlike human macrophages, *S. Typhi* cannot survive within macrophages of mice, a nonpermissive species (10, 11). The interaction of *S. enterica* with host cells is largely determined by the activities of two type III secretion systems (T3SS), which deliver bacterial effector proteins into host cells to modulate a variety of cellular processes (12–15). Differences in the assortment of the T3SS effector proteins encoded by *S. Typhi* and *S. Typhimurium* result in differences in the composition of the membrane compartment that harbors these bacteria (16). Whereas vacuoles containing *S. Typhi* recruit

Rab29, those containing the broad-host serovar *S. Typhimurium* do not, because Rab29 is degraded by the T3SS effector GtgE, absent from *S. Typhi* (16).

We hypothesized that the presence or absence of a Rab guanosine triphosphatase (GTPase) in the vacuoles harboring *S. Typhi* or *S. Typhimurium* must translate into fundamental differences in the intracellular biology of these pathogens, potentially influencing host-cell restriction. We expressed the *S. Typhimurium* T3SS effector GtgE in *S. Typhi* and examined its ability to survive within primary bone-marrow-derived macrophages (BMDMs) obtained from mice, a nonpermissive species (17). Surprisingly, expression of *gtgE* significantly increased the ability of *S. Typhi* to survive within mouse BMDMs (Fig. 1A and fig. S1). Whereas very few bacteria were recovered from mouse macrophages 48 hours after infection with wild-type *S. Typhi*, large numbers of bacteria were recovered from macrophages infected with *S. Typhi* expressing *gtgE*. Indeed, the number of colony-forming units (CFU) recovered from macrophages infected with *S. Typhi* expressing *gtgE* were equivalent to those recovered from macrophages infected with the broad-host serovar *S. Typhimurium* (Fig. 1A and fig. S1). Thus, expression of a single effector protein from broad-host *Salmonellae* allows *S. Typhi* to overcome host-cell restriction and survive in a nonpermissive host cell.

To investigate the consequences of overcoming host-cell restriction for the ability of *S. Typhi* to replicate within a nonpermissive host, we infected mice with the *S. Typhi* strain expressing *gtgE*. We found a significantly higher number of bacteria in the tissues of mice infected with *S. Typhi* expressing *gtgE* compared to those infected with wild type (Fig. 1, B and C, and fig. S2). Thus, expression of *gtgE* allows *S. Typhi* to overcome some of the host restriction barrier.

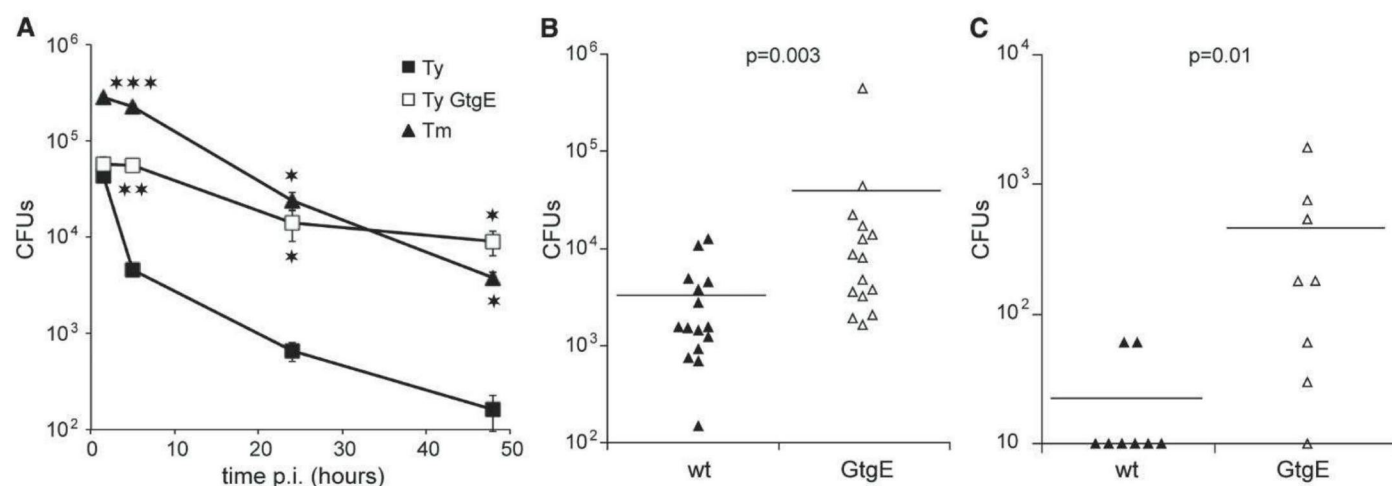


Fig. 1. GtgE expression limits host-cell restriction. (A) Survival of *S. Typhi* expressing GtgE in bone-marrow-derived mouse macrophages (BMDMs). Macrophages were infected with *S. Typhi* (Ty), *S. Typhi* expressing GtgE (Ty GtgE), or *S. Typhimurium* (Tm). Cells were lysed at the indicated time points and CFU enumerated. Values are means $\pm$ SEM of three independent measurements. *P* values (for the difference relative to values obtained from cells

infected with *S. Typhi*) were determined by the Student's *t* test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. (B and C) CFU recovered from the spleens of C57BL/6 Nrmpp^{+/+} mice infected with *S. Typhi* (wt, wild type) or *S. Typhi* expressing GtgE (GtgE) 4 days after intraperitoneal (B) or oral (C) inoculation. Horizontal bars indicate the means. The *P* value was determined by the Wilcoxon rank sum test.

Furthermore, the inability of *S. Typhi* to replicate within nonhuman hosts appears at least in part due to its inability to survive within macrophages of the nonpermissive species. However, additional factors must contribute to host restriction, because the virulence of *S. Typhi* expressing GtgE did not match that of *S. Typhimurium* (18), which is highly virulent for mice.

To gain insight into the mechanisms by which GtgE allows *S. Typhi* to overcome the host-cell restriction barrier, we examined the effect of depleting Rab29, a target of its protease activity (16), on the ability of *S. Typhi* to survive within primary mouse BMDMs. Surprisingly, small interfering RNA (siRNA)-mediated depletion of Rab29 had no effect on the ability of *S. Typhi* to survive in nonpermissive macrophages (fig. S3), which indicated that GtgE might have an additional cellular target(s) for its protease activity. We therefore examined the ability of GtgE to target other Rab GTPases. Despite the broad conservation of residues surrounding the GtgE cleavage site in several GTPases (fig. S4), GtgE could only target Rab32 and Rab38 (Fig. 2, A to C, and fig. S5), which are the GTPases most highly related to Rab29 (Fig. 2D and fig. S6). However, GtgE did not cleave Rab23, the next most highly related GTPase, nor did it cleave other GTPases phylogenetically close to Rab29, Rab32, or Rab38 (Fig. 2, A, C, and D, and figs. S4 to S6). Rab32

and Rab38 (but not Rab23) were cleaved in cells infected with *S. Typhimurium*, which expresses GtgE, but not in cells infected with *S. Typhi*, which does not (Fig. 2C). Thus, GtgE is a specific protease that targets a very restricted subgroup of highly related Rab GTPases.

Shortly after infection, Rab29 is recruited to the *S. Typhi*-containing vacuoles and remains associated with these vacuoles for several hours after infection (16). Like Rab29, Rab32 and Rab38 were recruited to the *S. Typhi*-containing vacuole with similar kinetics (Fig. 3A). Similar observations were made in cells infected with the related human-restricted serovar *Salmonella* Paratyphi, which, like *S. Typhi*, does not encode *gtgE* (Fig. 3B). In contrast, Rab32 and Rab38 were not recruited to the *S. Typhimurium* vacuole (Fig. 3B), consistent with the GtgE-mediated degradation of these GTPases in infected cells (Fig. 2C). Similar results were observed in mouse primary BMDMs (Fig. 3C). Thus, there are marked differences between the composition of the intracellular compartments that harbor the human-restricted *S. Typhi* and the broad-host *Salmonellae* such as *S. Typhimurium*, which may contribute to their differences in host specificity.

To ascertain which of the GtgE targets restricts the survival of *S. Typhi* within nonpermissive macrophages, we investigated the effect of siRNA-mediated depletion of Rab32 or Rab38

on the ability of *S. Typhi* to survive within primary mouse BMDMs. Depletion of Rab38 had no effect on the ability of *S. Typhi* to survive in nonpermissive macrophages (Fig. 4A and fig. S7). However, depletion of Rab32 significantly increased the ability of *S. Typhi* to survive within mouse macrophages, essentially phenocopying *S. Typhi* expressing *gtgE* (Fig. 4A and fig. S7). Thus, GtgE confers on *S. Typhi* the capacity to survive within mouse macrophages by degrading Rab32.

Rab32 and Rab38 have been implicated in the biogenesis of specialized compartments distinct from lysosomes that are collectively known as lysosome-related organelles (LROs) such as melanosomes or specialized granules in platelet and T cells (19–24). Rab32 and Rab38, in conjunction with BLOC-1, -2, and -3, coordinate the delivery of specific cargo to LROs including enzymes required for melanin synthesis or a variety of antimicrobial proteins (20, 23, 25, 26). Intriguingly, the *Salmonella*-containing vacuole (SCV) exhibits features of LROs such as the presence of the lysosomal glycoprotein 1 (Lamp-1), the absence of lysosomal degradative enzymes (27, 28) and, as shown here, the presence of Rab32 and Rab38 (fig. S8). Rab32 may therefore restrict *S. Typhi* growth in mouse macrophages by delivering an antimicrobial activity to the SCV. If this hypothesis is correct, interfering with components

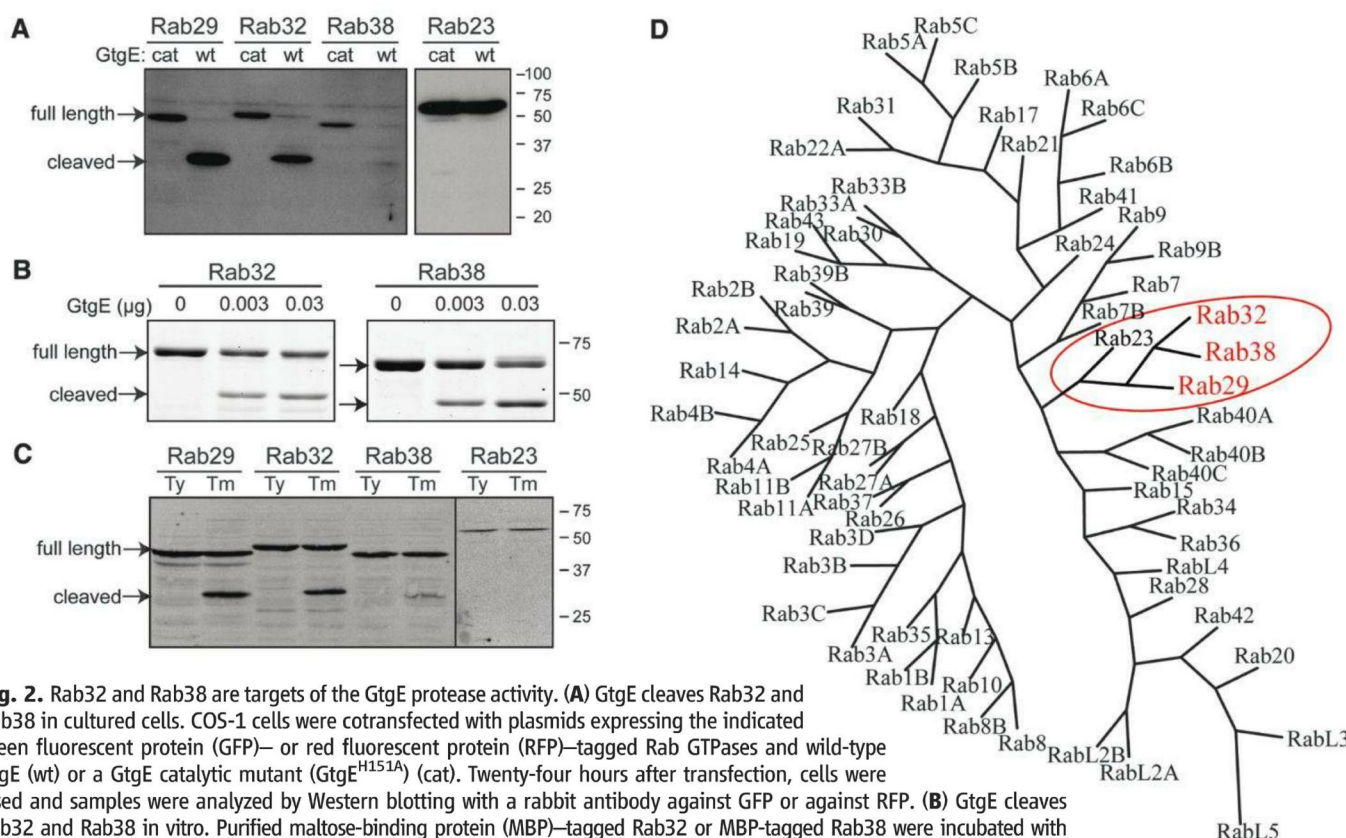


Fig. 2. Rab32 and Rab38 are targets of the GtgE protease activity. (A) GtgE cleaves Rab32 and Rab38 in cultured cells. COS-1 cells were cotransfected with plasmids expressing the indicated green fluorescent protein (GFP)- or red fluorescent protein (RFP)-tagged Rab GTPases and wild-type GtgE (wt) or a GtgE catalytic mutant (GtgE^{H151A}) (cat). Twenty-four hours after transfection, cells were lysed and samples were analyzed by Western blotting with a rabbit antibody against GFP or against RFP. (B) GtgE cleaves Rab32 and Rab38 in vitro. Purified maltose-binding protein (MBP)-tagged Rab32 or MBP-tagged Rab38 were incubated with MBP-tagged GtgE, separated by SDS-polyacrylamide gel electrophoresis, and stained with Coomassie. (C) GtgE targets Rab32 and Rab38 during *Salmonella* infection. COS-1 cells expressing GFP-Rab29, yellow fluorescent protein (YFP)-Rab32, YFP-Rab38, or RFP-Rab23 were infected with *S. Typhi* (Ty) or *S. Typhimurium* (Tm). Two and a half hours after infection, cells were lysed and samples were analyzed by Western blotting with a rabbit antibody against GFP or against RFP. (D) Phylogenetic tree of the human Rab and Rab-like GTPases. The locations of Rab29, Rab32, and Rab38 within the tree are indicated in red.

Fig. 3. Rab32 and Rab38 are recruited to the *Salmonella* Typhi-containing vacuole. Henle-407 cells (A and B) or mouse primary BMDMs (C) expressing YFP- or CFP-tagged Rab32 or Rab38 (green) were infected with *S. Typhi* (A and C), *S. Paratyphi* (B and C), or *S. Typhimurium* (B and C) expressing mCherry (red) and imaged at the indicated times (A) or 2 hours (B and C) after infection. The images shown represent maximum intensity projections of Z-stacks. Bars, 10 μ m.

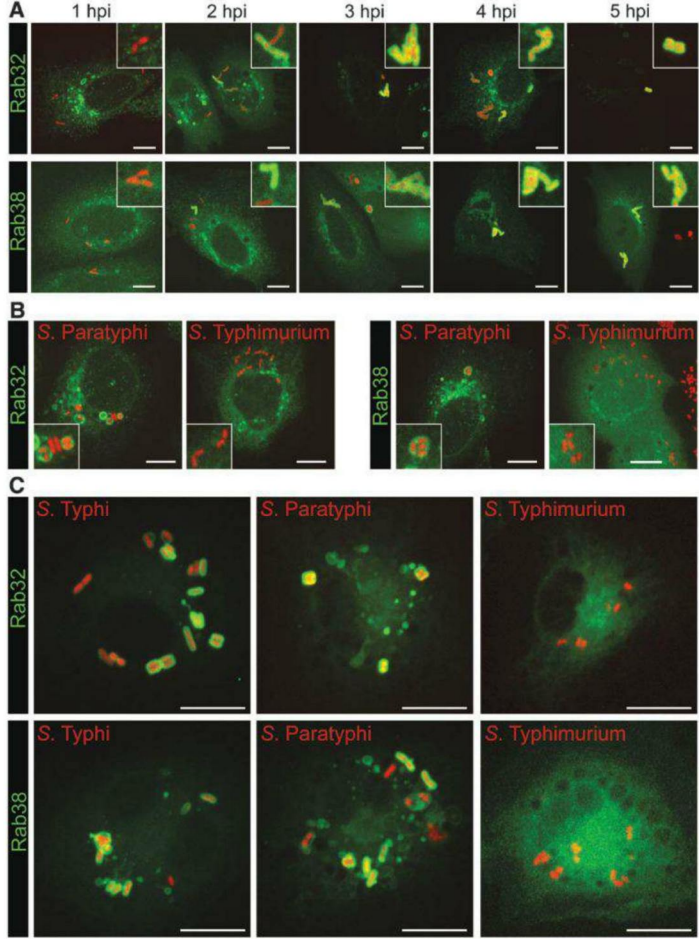
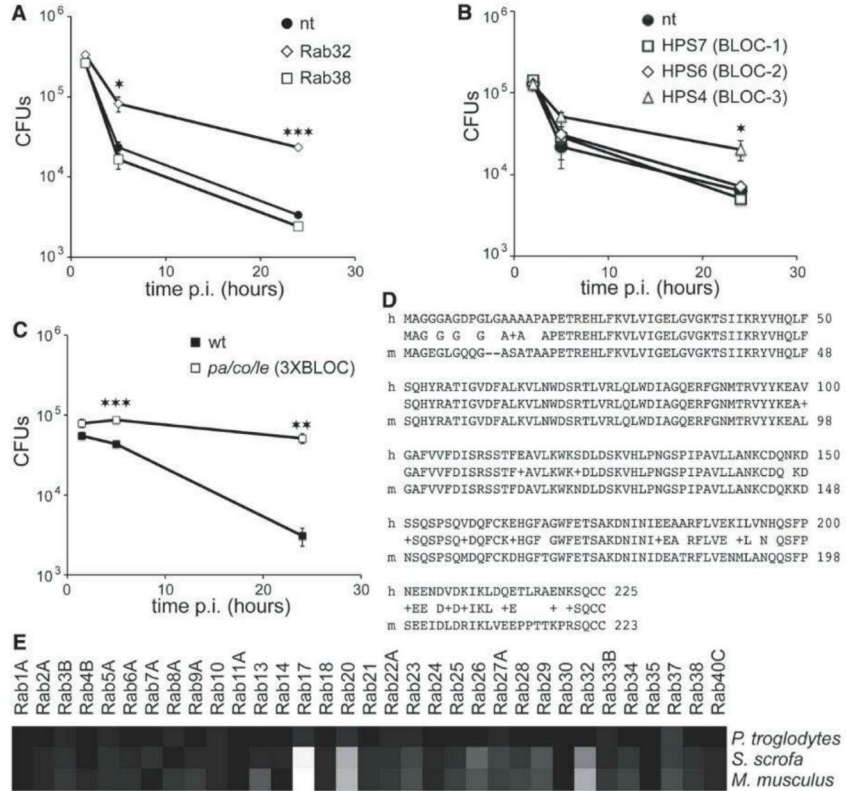


Fig. 4. Rab32 and BLOC-3 are required for *S. Typhi* host-cell restriction. (A) Intracellular survival of *S. Typhi* in BMDMs depleted of Rab32 and Rab38. BMDMs from mice were transfected with a nontargeting siRNA smart pool (nt) or siRNA smart pools targeting Rab32 or Rab38. Three days after transfection, cells were infected with *S. Typhi* and then lysed at the indicated time points after infection, and CFU were enumerated. Values are means $\pm$ SEM of three independent measurements. (B) Intracellular survival of *S. Typhi* in BMDMs depleted of BLOCs. BMDMs from mice were nucleoporated with a nontargeting siRNA smart pool (nt) or specific siRNA smart pools targeting HPS7 (subunit of BLOC-1), HPS6 (subunit of BLOC-2), or HPS4 (subunit of BLOC-3). Three days after transfection, cells were infected with *S. Typhi* and then lysed at the indicated time points, and CFU were enumerated. Values are means $\pm$ SEM of three independent experiments. (C) Intracellular survival of *S. Typhi* in BMDMs defective for BLOC-1, -2, and -3. BMDM cells from mice simultaneously defective in BLOC-1, 2, and -3 (*pa/co/le*) were infected with *S. Typhi* and then lysed at the indicated time points after infection, and CFU were enumerated. Values are means $\pm$ SEM of three independent measurements. (D) Alignment of human (h) and mouse (m) Rab32 shows sequence variation in the N-terminal and C-terminal regions. (E) Heat-map of the degrees of identity of the human Rab GTPases with the orthologs in three mammalian species: *Pan troglodytes*, *Sus scrofa*, *Mus musculus*. Black represents 100% identity, and white 75% identity. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 (Student's *t* test).



of the BLOC complexes should allow *S. Typhi* to survive in mouse macrophages. Depletion of HPS-7 or HPS-6, essential components of BLOC-1 and BLOC-2, respectively, did not affect the ability of *S. Typhi* to survive within mouse macrophages (Fig. 4B and fig. S9). In contrast, depletion of HPS-4, an essential component of BLOC-3, allowed *S. Typhi* to survive within mouse macrophages (Fig. 4B and fig. S9). Furthermore, *S. Typhi* survived in BMDMs obtained from a mouse (*pa/co/le*) simultaneously defective in BLOC-1, -2, and -3 (29–31) (Fig. 4C). Thus, *S. Typhi* restriction in cells from a nonpermissive host is due to an antimicrobial activity delivered to its intracellular vacuole by a machinery akin to that used in the genesis of specialized compartments such as melanosomes and T cell granules. The rapid loss of *S. Typhi* CFU after infection of nonpermissive cells (Fig. 1) suggests that this activity must kill *S. Typhi* rather than restrict its growth. Perhaps differences in the antimicrobial activity of human macrophages may have relieved *S. Typhi* from the need to acquire (or retain) *gtgE*, thus contributing to its host specialization.

Rab GTPases are highly conserved across vertebrate species (32). However, Rab32 exhibits relatively more amino acid sequence variation across mammalian species than most other members of this family (Fig. 4, D and E). This variation may have been driven by the action of virulence factors that, like GtgE, may target this host-defense pathway. Mutations in the components of the machinery involved in melanosome

formation such as the BLOCs or a geranylgeranyl transferase that modifies Rab32 lead to a variety of pathologies such as Hermansky Pudlak syndrome (33, 34). These deficiencies lead not only to albinism but also to other clinical manifestations, including increased susceptibility to infections (35). Deficiencies in macrophage microbial killing functions may contribute to the observed increased susceptibility to bacterial infections. Indeed, a recent genome-wide association study has uncovered a genetic polymorphism in Rab32 that is linked to increased susceptibility to *Mycobacterium leprae* infection (36). Furthermore, Rab32 has been reported to be present in the *Mycobacterium tuberculosis*-containing vacuole (37). It is thus likely that the mechanism described here may be important in the control of other intracellular pathogens that, like *Salmonella*, reside in this specialized membrane compartment.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S9

Table S1

References (38–42)

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Salmonella Inhibits Retrograde Trafficking of Mannose-6-Phosphate Receptors and Lysosome Function

Kieran McGourty,¹ Teresa L. Thurston,¹ Sophie A. Matthews,¹ Laurie Pinaud,^{1*} Luís Jaime Mota,^{1†} David W. Holden^{1‡}

Salmonella enterica is an intracellular bacterial pathogen that replicates within membrane-bound vacuoles through the action of effector proteins translocated into host cells. *Salmonella* vacuoles have characteristics of lysosomes but are reduced in hydrolytic enzymes transported by mannose-6-phosphate receptors (MPRs). We found that the effector SifA subverted Rab9-dependent retrograde trafficking of MPRs, thereby attenuating lysosome function. This required binding of SifA to its host cell target SKIP/PLEKHM2. Furthermore, SKIP regulated retrograde trafficking of MPRs in noninfected cells. Translocated SifA formed a stable complex with SKIP and Rab9 in infected cells. Sequestration of Rab9 by SifA-SKIP accounted for the effect of SifA on MPR transport and lysosome function. Growth of *Salmonella* increased in cells with reduced lysosomal activity and decreased in cells with higher lysosomal activity. These results suggest that *Salmonella* vacuoles undergo fusion with lysosomes whose potency has been reduced by SifA.

Lysosomes are membrane-bound acidic organelles that contain over 50 different hydrolytic enzymes. In the trans-Golgi network (TGN), the majority of newly synthesized hydrolytic enzymes bind the cation-dependent (CD-) and cation-independent (CI-) mannose phosphate receptors (MPRs), which then deliver the hydrolases to endosomes. Endosome acidification dissociates the hydrolases from their

MPRs, which recycle back to the TGN. As early endosomes mature into late endosomes, the hydrolases are converted into their active forms and transported to lysosomes (1). Lysosomes fuse with vacuoles containing microbes and other exogenous material, causing their degradation.

Salmonella enterica proliferates within mammalian cells in membrane-bound compartments: *Salmonella*-containing vacuoles (SCVs) (2). SCVs

interact extensively with the endocytic pathway, resulting in acidic compartments (3) whose membranes are enriched in lysosomal membrane glycoproteins (4, 5) and which remain accessible to late endosomal and lysosomal content (6). However, the CD- and CI-MPRs, as well as their associated hydrolases, do not accumulate in SCVs (4, 5, 7, 8). To investigate this paradox, we studied the consequences of *Salmonella* infection on the distribution of MPRs. Human epithelial (HeLa) cells were infected with wild-type *S. enterica* serovar Typhimurium (*Salmonella*), and the amounts of TGN-localized CD-MPR and CI-MPR were quantified by three-dimensional confocal microscopy. In uninfected cells, the proteins accumulated as expected at the TGN (Fig. 1A). In infected cells, the CD- and CI-MPRs were distributed diffusely throughout the cell (Fig. 1, A and B), whereas the localization of other TGN-associated proteins was unaffected (fig. S1, A and B).

Salmonella translocates effector proteins across the SCV membrane via the *Salmonella* pathogenicity island 2 (SPI-2)-encoded type III secretion

¹Section of Microbiology, Centre for Molecular Microbiology and Infection, Imperial College London, Armstrong Road, London SW7 2AZ, UK.

*Present address: Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur, 25–28 Rue du Docteur Roux, 75015 Paris, France.

†Present address: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da República, 2780-157 Oeiras, Portugal.

‡To whom correspondence should be addressed. E-mail: d.holden@imperial.ac.uk

system (T3SS). SPI-2 T3SS effectors mediate intravacuolar bacterial replication (2). HeLa cells infected with an *ssaV* mutant strain that has a nonfunctional T3SS (9) were indistinguishable from uninfected cells in terms of the distribution of MPRs (Fig. 1, A and B). The reduction of MPR from the TGN began 6 to 8 hours after bacterial invasion (fig. S1D), when other effects of the SPI-2 T3SS become visible (10). Thus, the SPI-2 T3SS is required for the redistribution of MPRs from the TGN. Two endocytic-TGN recycling pathways have been described: One depends on the Q-soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor (Q-SNARE)

syntaxin 6 (Syn6) and transports TGN46 and the cholera toxin B subunit (CTxB); the other depends on the Q-SNARE syntaxin 10 (Syn10) and involves retrograde transport of MPRs (11). Occasionally the CI-MPR is transported to the plasma membrane from the TGN (12). To determine whether intracellular *Salmonella* interferes with these pathways, we assayed the transport of fluorescent CTxB or an antibody against CI-MPR from the plasma membrane to the TGN in infected cells. Knockdown of Syn6 inhibited transport of the CTxB to the TGN, but there was no detectable effect of *Salmonella* on this pathway (Fig. 1C). In contrast, trafficking of CI-MPR anti-

body was dramatically reduced in cells infected with wild-type *Salmonella* (Fig. 1, D and E, and fig. S2A), which depended on a functional SPI-2 T3SS (Fig. 1D). Thus, the SPI-2 T3SS specifically interferes with Syn10-dependent retrograde trafficking of MPRs from endosomes to the TGN.

Interference of MPR recycling leads to misrouted secretion of newly synthesized lysosomal enzymes (13). We examined the effect of intracellular *Salmonella* on the secretion of two lysosomal enzymes: cathepsin D (CatD) and β -hexosaminidase (β -hexo). Infection of HeLa cells with wild-type *Salmonella* significantly increased

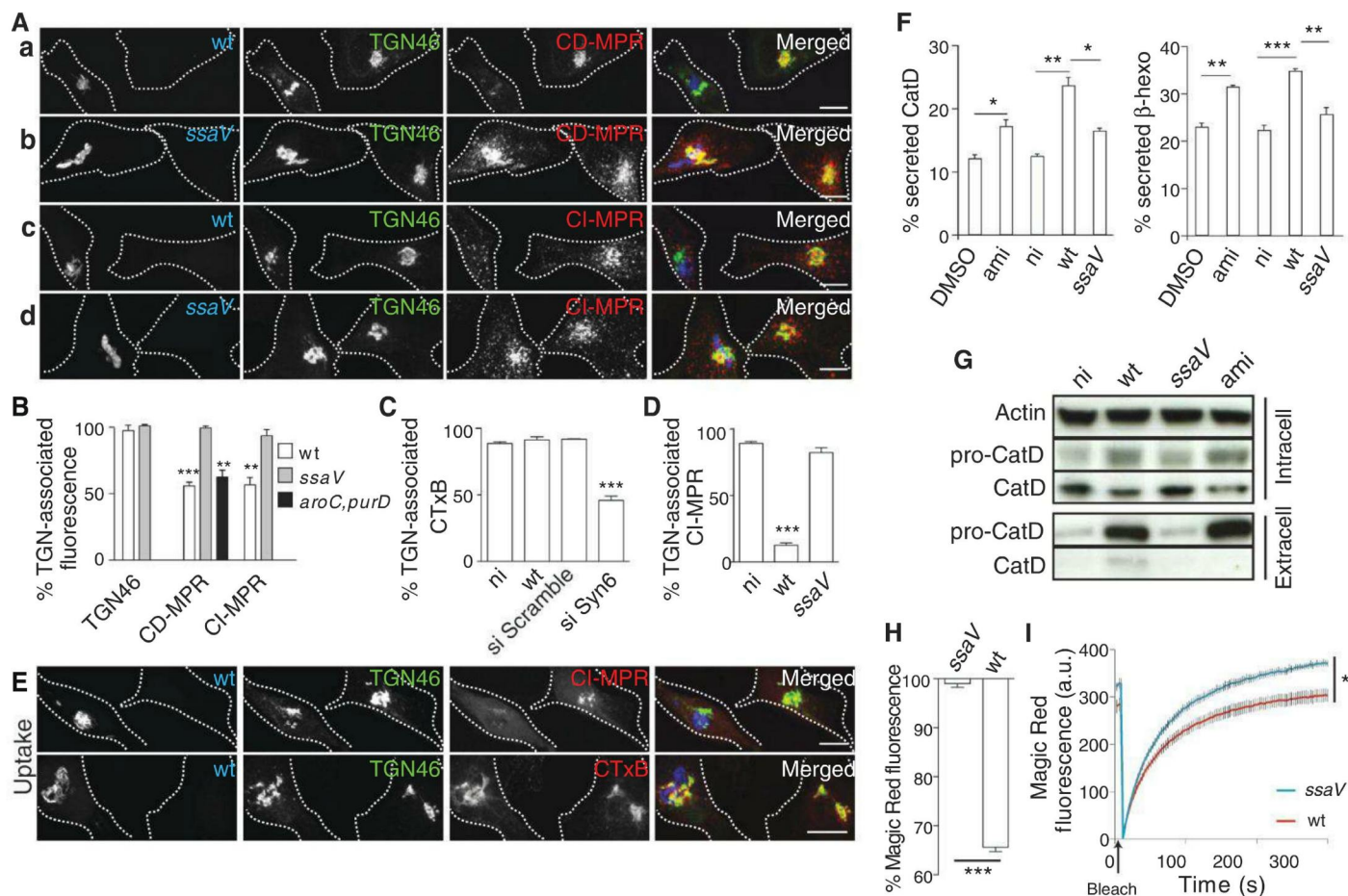


Fig 1. The SPI-2 T3SS interferes with Syn10-dependent retrograde trafficking of MPRs and reduces lysosome function. (A and B) HeLa cells were infected for 14 hours with green fluorescent protein (GFP)-expressing wild-type (wt) (Aa and c) or *ssaV* mutant (Ab and d) *Salmonella* (blue) or (B) a replication-deficient *aroC, purD* mutant (23) that has a functional SPI-2 T3SS (fig. S1C). Cells were immunolabeled for TGN46 (green) and CD-MPR (red) (a and b) or CI-MPR (red) (c and d). Scale bars, 10 μ m. (B) Quantitation of mean fluorescent signals of TGN-associated TGN46 (24), CD-MPR, and CI-MPR, analyzed by three-dimensional (3D) confocal microscopy and normalized to signals in uninfected cells from the same sample. Error bars in all figures indicate SEM. (C to E) Plasma membrane-TGN transport assays. HeLa cells either were subjected to siRNA to deplete Syn6 or infected with wt or *ssaV* mutant *Salmonella* (blue in E) for 14 hours, then pulse-chased with either fluorescent CTxB [red in (E)] or CI-MPR antibody [red in (E)]. Fixed cells were immunolabeled for CI-MPR and TGN46 [green in (E)] or Rab6 (to identify the TGN in si Syn6- and si Scramble-treated cells), and TGN-associated CI-MPR or CTxB was quan-

tified. Scale bars, 10 μ m. (F and G) Rerouting of lysosomal enzymes. (F) HeLa cells were exposed to dimethyl sulfoxide (DMSO) or amiodarone (ami) [which redistributes MPRs (13)] and were not infected (ni) or infected with wt or *ssaV* mutant *Salmonella* for 14 hours. Secreted levels of cathepsin D (CatD) or β -hexosaminidase (β -hexo) are a percentage of total levels. (G) Intra- and extracellular proteins were analyzed by immunoblotting, using actin as a loading control. (H and I) Cathepsin B (CatB) activity was quantified by measuring fluorescent cleavage product of Magic Red-RR in living cells. (H) HeLa cells were infected for 14 hours with wt or *ssaV* mutant *Salmonella* and exposed to Magic Red-RR, and steady-state fluorescent product intensity was measured by flow cytometry. Bars represent the percentage of fluorescence of infected compared with uninfected cells in the same sample. (I) Cells as in (H) were photobleached and analyzed for new fluorescent product. Data were normalized to the mean fluorescence of uninfected cells. Vertical lines represent SEM for each of 100 time points. Statistically significant relationships are denoted (* P < 0.05; ** P < 0.01; *** P < 0.005).

extracellular activities of both enzymes compared with uninfected or untreated cells. The SPI-2 T3SS accounted for a major proportion of the *Salmonella* effect (Fig. 1F). Infection of cells by wild-type bacteria caused the appearance in the extracellular medium of large amounts of immature CatD (Fig. 1G), showing that it had not been processed in an endosomal environment. This was accompanied by a reduction in the level of intracellular mature enzyme and required an intact SPI-2 T3SS (Fig. 1G).

A misrouting of lysosomal enzymes would be expected to cause a reduction of lysosome function. We tested the effect of *Salmonella* infection and the SPI-2 T3SS on the activity of lysosomal cathepsin B (CatB) in living HeLa cells by using a substrate (Magic Red-RR, ImmunoChemistry Technologies, Bloomington, Minnesota) that becomes fluorescent upon cleavage (14). Cells infected with *ssaV* mutant bacteria had higher steady-state

levels of fluorescence (Fig. 1H) and recovered to higher levels after photobleaching compared with cells infected with wild-type *Salmonella* (Fig. 1I and movies S1 and S2). A reduction in lysosomal CatB activity was also observed after small interfering RNA (siRNA)-mediated depletion of Syn10 in uninfected HeLa cells (fig. S3, A and B), whereas siRNA-mediated depletion of Syn6 enhanced CatB activity (fig. S3, A and B), which correlated with reduced and enhanced MPR localization at the TGN, respectively (fig. S3C).

SifA is a *Salmonella* effector required to maintain the SCV membrane (10). Loss of the vacuolar membrane around a *sifA* mutant is inhibited by absence of another effector, SseJ (15). TGN-associated CD-MPR was reduced by infection with the *sseJ* mutant or wild-type strain but not by the *sifA,sseJ* or *ssaV* mutant strains (Fig. 2A). SifA interacts with a host protein, SifA

and kinesin-interacting protein (SKIP)/pleckstrin homology domain containing, family M (with RUN domain) member 2 (PLEKHM2), to regulate kinesin activity and vacuolar membrane dynamics (16, 17). This interaction is dependent on leucine at position 130 (L130) of SifA (18). HeLa cells were infected with the *sifA,sseJ* mutant strain harboring a plasmid encoding either a hemagglutinin epitope-tagged wild-type SifA (SifA-HA) or a point mutant (SifA_{L130D}-HA). Cells infected with *Salmonella* expressing wild-type SifA but not SifA_{L130D} showed redistribution of MPR (Fig. 2, A and B). Similarly, lysosomal CatB activity was consistently lower in cells infected with either wild-type bacteria or with the *sifA,sseJ* mutant expressing SifA-HA compared with cells infected with the *sifA,sseJ* mutant or with this strain expressing SifA_{L130D}-HA (Fig. 2, C and D). Cells infected with the *sifA,sseJ* mutant also had significantly higher lysosomal activity than wild-type-infected

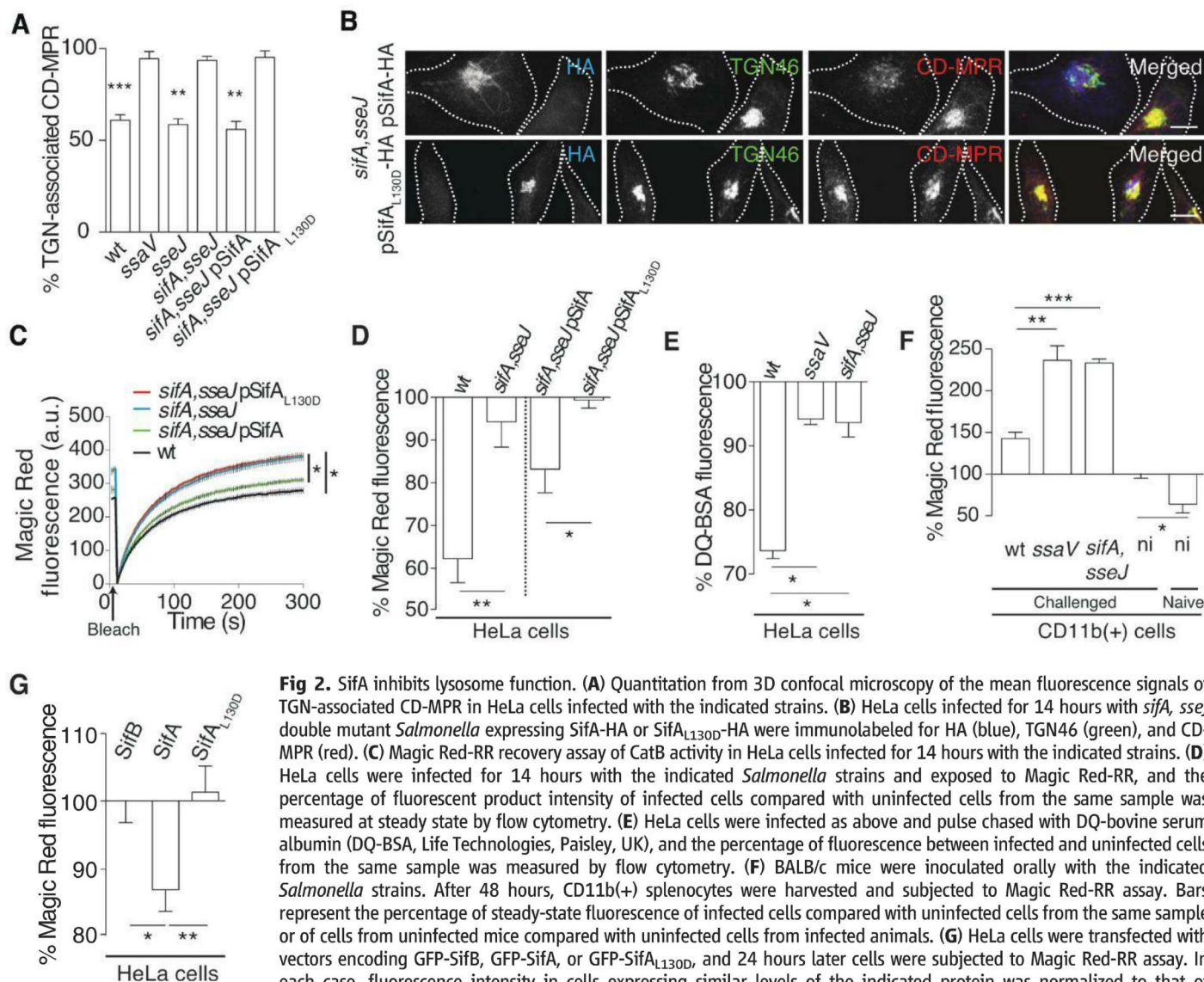


Fig 2. SifA inhibits lysosome function. (A) Quantitation from 3D confocal microscopy of the mean fluorescence signals of TGN-associated CD-MPR in HeLa cells infected with the indicated strains. (B) HeLa cells infected for 14 hours with *sifA,sseJ* double mutant *Salmonella* expressing SifA-HA or SifA_{L130D}-HA were immunolabeled for HA (blue), TGN46 (green), and CD-MPR (red). (C) Magic Red-RR recovery assay of CatB activity in HeLa cells infected for 14 hours with the indicated strains. (D) HeLa cells were infected for 14 hours with the indicated *Salmonella* strains and exposed to Magic Red-RR, and the percentage of fluorescent product intensity of infected cells compared with uninfected cells from the same sample was measured at steady state by flow cytometry. (E) HeLa cells were infected as above and pulse chased with DQ-bovine serum albumin (DQ-BSA, Life Technologies, Paisley, UK), and the percentage of fluorescence between infected and uninfected cells from the same sample was measured by flow cytometry. (F) BALB/c mice were inoculated orally with the indicated *Salmonella* strains. After 48 hours, CD11b(+) splenocytes were harvested and subjected to Magic Red-RR assay. Bars represent the percentage of steady-state fluorescence of infected cells compared with uninfected cells from the same sample or of cells from uninfected mice compared with uninfected cells from infected animals. (G) HeLa cells were transfected with vectors encoding GFP-SifB, GFP-SifA, or GFP-SifA_{L130D}, and 24 hours later cells were subjected to Magic Red-RR assay. In each case, fluorescence intensity in cells expressing similar levels of the indicated protein was normalized to that of untransfected control cells within the same sample and then to fluorescence caused by expression of SifB. Statistically significant relationships are denoted (* P < 0.05; ** P < 0.01; *** P < 0.005).

cells (Fig. 2E). SPI-2 T3SS-dependent inhibition of lysosome function was also observed in mouse-derived macrophages (fig. S4) and in CD11b(+) splenocytes from infected mice (Fig. 2F). Ectopic expression of SifA in HeLa cells significantly reduced TGN-localized CD-MPR (fig. S5A) and lysosomal activity when compared with cells expressing similar levels of SifA_{L130D} or another effector, SifB (Fig. 2G and fig. S5B). Thus, SifA accounts for the inhibition of lysosomal function by *Salmonella* and the requirement for L130 implicates SKIP in this process.

siRNA-mediated knockdown of SKIP (fig. S6A) resulted in an increase in both localization of the CD-MPR at the TGN (Fig. 3A) and CatB activity (Fig. 3, B and C). Immortalized mouse embryonic fibroblasts (MEFs) from SKIP knock-out mice (fig. S6B) displayed significantly reduced CatB activity when transfected with a vector encoding wild-type SKIP compared with cells expressing a SKIP variant lacking its SifA-binding pleckstrin homology (PH) domain (N-550) (Fig. 3D and fig. S6, C and D). To investigate the possible involvement of SKIP in endosome-TGN trafficking, we subjected HeLa cells depleted of SKIP to retrograde transport assays. Depletion of SKIP had no detectable effect on traffic of CTxB from the plasma membrane to the TGN (Fig. 3E), but there was a significant increase in the amount of CI-MPR transported to the TGN (Fig. 3E and fig. S2B), indicating that SKIP negatively regulates the Syn10-dependent retrograde pathway and lysosome function. To determine whether the effect of SifA on lysosome function required SKIP, wild-type and *Skip*^{-/-} knockout MEFs expressing either SifA or SifB were assayed for CatB activity. Compared with SifB, SifA reduced enzyme activity in wild-type MEFs but not in *Skip*^{-/-} knockout cells (Fig. 3F). Thus, the inhibitory activity of SifA can be attributed to a pathway requiring SKIP.

MPR levels were reduced in *Salmonella*-infected HeLa cells (fig. S7A), and a significant proportion of the remaining protein relocated to structures containing Vps26 (fig. S7B), a component of the retromer complex that mediates sorting of cargo from endosomes before trafficking to the TGN (11, 19). Because there was no effect on the steady-state localization of Syn10 at the TGN (fig. S8), these observations suggested that *Salmonella* interferes with the Syn10 pathway downstream of retromer sorting and upstream of the Syn10-containing SNARE complex that mediates vesicle fusion at the TGN (11). The small guanosine triphosphatase (GTPase) Rab9 is required for late endosome-TGN transport of MPRs (20, 21). It also binds to the Golgi-associated protein GCC185, which tethers MPR-containing vesicles and enables their fusion at the TGN (11). The proportion of Rab9 colocalizing with CD-MPR was unaffected in HeLa cells infected with the *sifA*, *sseJ* mutant but significantly reduced in cells infected with wild-type bacteria. In contrast, there was significantly greater colocalization between these proteins in un-

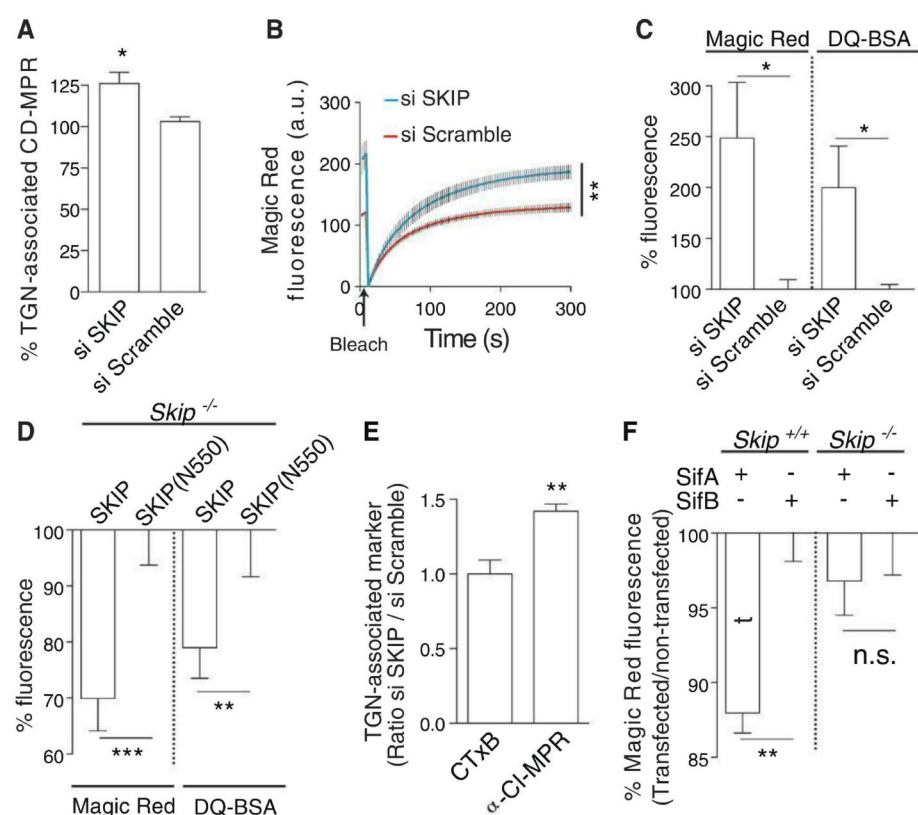


Fig 3. SKIP negatively regulates lysosome function. (A and B) HeLa cells depleted of SKIP by siRNA or exposed to scrambled siRNA were analyzed for TGN-associated CD-MPR (A) and by Magic Red-RR assay of recovery of CatB activity after photobleaching (B). (C) Steady-state levels of CatB activity and DQ-BSA hydrolysis analysed by flow cytometry. For each substrate, the data were normalized to that obtained by using scrambled siRNA. (D) *Skip*^{-/-} MEFs expressing either an N-terminal domain of SKIP (N550) or full-length protein were analyzed for steady-state levels of CatB by Magic Red-RR assay. Data are normalized to SKIP (N550) samples. (E) HeLa cells were depleted of SKIP by siRNA (si SKIP) or exposed to scrambled siRNA and subjected to plasma membrane-TGN transport assays using fluorescent CTxB or CI-MPR antibody. Data were analyzed by 3D confocal microscopy and were normalized to that obtained by using scrambled siRNA. (F) Wild-type (*Skip*^{+/+}) or *Skip*^{-/-} MEFs were transfected with vectors encoding either GFP-SifA or GFP-SifB and assayed for CatB activity by Magic Red-RR. Bars represent the percentage of fluorescence of transfected cells compared with untransfected cells from the same samples, normalized to GFP-SifB in either *Skip*^{+/+} or *Skip*^{-/-} cells. Statistically significant relationships are denoted (**P* < 0.05; ***P* < 0.01; ****P* < 0.005).

infected cells depleted of SKIP (Fig. 4A). Thus, SKIP appears to negatively regulate interactions between compartments containing MPR and Rab9, and SifA might enhance this by binding to SKIP.

To investigate possible interactions between SifA, SKIP and Rab9 in infected cells, we infected stable cell lines expressing GFP-Rab9 and Flag-SKIP with *Salmonella* expressing SifA-HA or SifA_{L130D}-HA. There was strong L130-dependent co-localization between SifA, SKIP, and Rab9 (Fig. 4B). Furthermore, SifA, SKIP, and Rab9 formed a stable complex in infected cells (fig. S9) that was dependent on L130 of SifA (Fig. 4C). Thus, SifA and SKIP appear to act as a “sink” for Rab9, thereby inhibiting MPR traffic and lysosome function (Fig. 4D). Indeed, overexpression of constitutively active Rab9 significantly impaired the ability of *Salmonella* to reduce lysosome function (fig. S10).

This work provides an explanation for the long-standing paradox that SCVs are relatively devoid of hydrolytic lysosomal enzymes (4) and yet retain lysosomal membrane glycoproteins in the vacuolar membrane and interact dynamically with the endolysosomal system (6). If *Salmonella* were exposed to lysosomal content, then its intracellular growth would be predicted to be sensitive to lysosome potency. Indeed, depletion of Syn6 (which augmented lysosome function; fig. S3B) resulted in reduced bacterial growth, whereas depletion of Syn10 (which decreased lysosome function; fig. S3B) or pharmacological inhibition of cathepsins resulted in increased bacterial growth (Fig. 4E and fig. S11). The actions of SPI-2 T3SS effectors that regulate SCV membrane dynamics (22) could promote interactions between SCVs and enzyme-depleted lysosomes to provide a source of vacuolar membrane and possibly nutrients to enable bacterial growth.

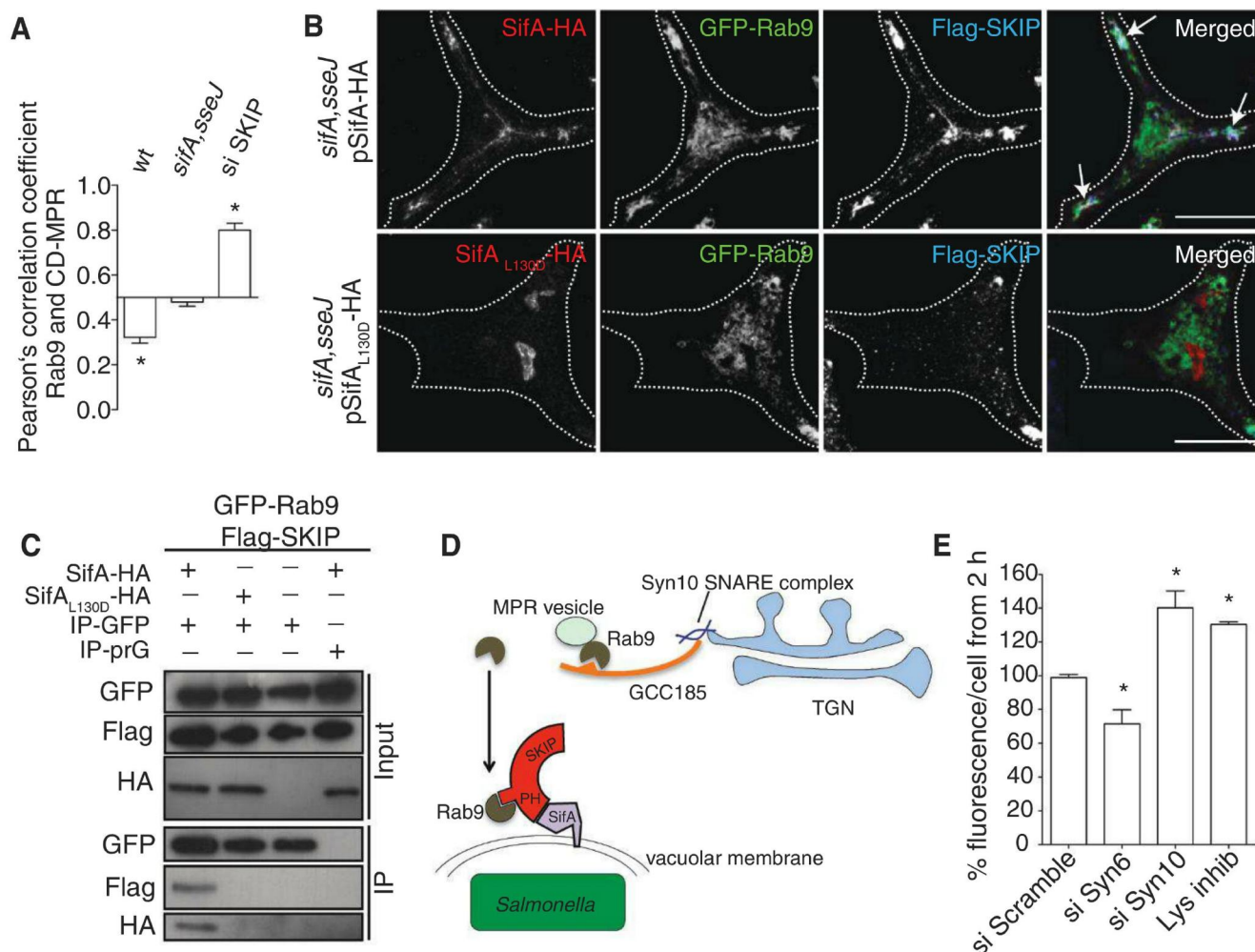


Fig 4. Interactions between SifA, SKIP, and Rab9. **(A)** HeLa cells depleted of SKIP (si SKIP) or infected for 14 hours with wt or *sifA, sseJ* double mutant *Salmonella* were fixed, labeled, and analyzed by quantitative confocal immunofluorescence microscopy. Pearson's correlation coefficient was calculated between Rab9 and CD-MPR and compared to noninfected or si Scramble-treated cells. **(B)** HeLa cells stably expressing Flag-SKIP and GFP-Rab9 were infected for 14 hours with *sifA, sseJ* mutant *Salmonella* expressing either SifA_{wt}-HA or SifA_{L130D}-HA. Cells were fixed, labeled, and analyzed by confocal immunofluorescence microscopy. Arrows indicate co-localization of the three proteins. **(C)** Cells infected as in **(B)** were lysed and proteins were immunoprecipitated with GRP antibody-conjugated beads, or protein G (prG)-beads as

a control. GFP-Rab9, SifA-HA, SifA_{L130D}-HA, and Flag-SKIP were detected in input samples (input) and after immunoprecipitation (output) by means of SDS-polyacrylamide gel electrophoresis and immunoblotting. **(D)** Model depicting interference of Rab9-mediated retrograde transport of MPR to the TGN by sequestration of Rab9 by SifA and SKIP. **(E)** HeLa cells treated with scramble, Syn6, or Syn10 oligos or with lysosome inhibitor were infected with GFP-expressing wild-type *Salmonella*. Bacterial load, measured as GFP fluorescence per HeLa cell, was measured by flow cytometry ($n > 10,000$). The % fluorescence/cell represents the ratio between the median GFP fluorescence at 2 and 8 hours after infection, normalized to mock-transfected or DMSO-treated cells. Statistically significant relationships are denoted (* $P < 0.05$).

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Supplementary Materials

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Materials and Methods
Figs. S1 to S11
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Movies S1 and S2

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Convergent Evolution Between Insect and Mammalian Audition

Fernando Montealegre-Z,^{1*}† Thorin Jonsson,¹ Kate A. Robson-Brown,² Matthew Postles,¹ Daniel Robert^{1*}

In mammals, hearing is dependent on three canonical processing stages: (i) an eardrum collecting sound, (ii) a middle ear impedance converter, and (iii) a cochlear frequency analyzer. Here, we show that some insects, such as rainforest katydids, possess equivalent biophysical mechanisms for auditory processing. Although katydid ears are among the smallest in all organisms, these ears perform the crucial stage of air-to-liquid impedance conversion and signal amplification, with the use of a distinct tympanal lever system. Further along the chain of hearing, spectral sound analysis is achieved through dispersive wave propagation across a fluid substrate, as in the mammalian cochlea. Thus, two phylogenetically remote organisms, katydids and mammals, have evolved a series of convergent solutions to common biophysical problems, despite their reliance on very different morphological substrates.

Many animals have evolved auditory organs with highly sensitive micro- and nanoscopic mechanisms that are able to detect vanishingly weak acoustic energy (1) and perform frequency analysis (2). In mammals, the chain of auditory biophysical events starts with the transformation of airborne acoustic energy into the mechanical vibrations of an eardrum. The lever action of delicate middle ear bones passes these eardrum vibrations to the oval window (Fig. 1), generating force gain via surface area ratio. This is the critically important step of impedance conversion that enables the efficient transfer of acoustic energy from airborne vibrations to the liquid-immersed mechanosensory hair cells in the cochlea (2). A second salient feature of many auditory systems is their capacity to analyze the frequency content of incoming sound waves. This process makes use of the mechanical anisotropy of the fluid-bathed basilar membrane to spatially decompose the acoustic signal into its frequency components, a biological form of the Fourier transform (3). Cochlear hair cells receive mechanical inputs at specific frequencies, depending on their position along the stiffness gradient of the basilar membrane (4, 5). This “piano keyboard” mapping, or tonotopic organization, is the canonical mechanism for frequency selectivity in mammals (5).

Impedance conversion is crucial to hearing in terrestrial mammals, yet it is unknown in insects. Here, we identify and characterize auditory mechanisms in an insect that are markedly convergent with those of mammalian ears. We studied the South American rainforest katydid *Copiphora*

gorgonensis (Orthoptera: Tettigoniidae: Copiphorini) and show that impedance transformation arises from unconventional tympanal mechanics, relying on a lever and fulcrum system and a favorable surface-area ratio to amplify and drive vibrations into the auditory sensory organ (Fig. 1). Next, we show that frequency analysis is enabled by the action of a newly identified organ, a fluid-filled vesicle, joining with the mechanosensory organ to support dispersive wave propagation and tonotopy. Our results reveal a notable case of convergence, whereby organisms with the most remote phylogenetic histories (such as mammals and katydids), have evolved to hear in a markedly analogous way.

As in all katydids, the ears of *C. gorgonensis* are located on the prothoracic tibia; the male calling song is a 23-kHz tone, with some frequency modulation (FM) (Fig. 2A) (6). Spanning only 600 μm , these ears are among the smallest known in all animals (7). X-ray microcomputer tomography (μCT ; 2.8- μm voxel resolution) of fresh

specimens reveals external and internal auditory anatomy. Each ear presents a pair of tympanal membranes (TMs) backed by an air-filled tracheal pipe connecting through the leg to the first spiracle on each side of the body (Fig. 2C and fig. S1) (8, 9). The mechanosensory organ, the crista acustica (CA), lies on the dorsal wall of the anterior tracheal branch (10, 11) and contains 28 mechanoreceptor units (Fig. 2D and fig. S1). In contrast to other auditory insects, such as locusts, flies, and moths (7, 12), katydid mechanoreceptors are not in direct contact with the TMs. In previous studies, the general morphological organization of the CA was found to be reminiscent of an uncoiled tapering basilar membrane (Fig. 2C) (10, 11, 13).

Here, we identify an organ in the katydid's ear: the acoustic vesicle (AV). The AV is derived from the hemolymphatic system, yet it is distinctly different. The AV is elongated and tapering, just like an uncoiled mammalian cochlea (Fig. 2, C and E). Preliminary apolar extraction (14) reveals that the AV contains a lipidic fluid, which is incompressible and would be suitable for high-velocity sound propagation. We have also shown that the AV is a functionally important organ in other katydid species (movie S1). The AV is located underneath the dorsal exocuticle of the tibia, adjacent to CA and between both TMs (Fig. 2, C and D), and connects with the hemolymph channel by a narrow constriction (Fig. 2, C to E). Notably, the AV is absent from both meso- and metathoracic legs, where no ears are found (fig. S2). Previous studies did not distinguish the AV from the conventional hemolymphatic supply (10, 13).

We used laser Doppler vibrometry (LDV) to examine the vibrational behavior of the proximal tibia (Fig. 2E) and to establish the functional importance of morphological components linking the TM to the CA (14). First, we measured tympanal mechanics in response to controlled

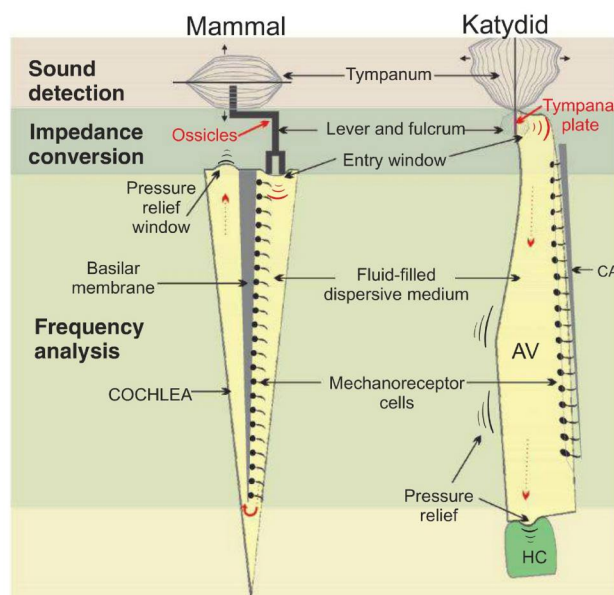


Fig. 1. Convergent auditory mechanisms between mammals and katydids. The three processing stages: (i) sound capture by the TM, (ii) impedance conversion in the middle ear, and (iii) frequency analysis in a fluid-filled dispersive medium. HC, hemolymph channel.

¹School of Biological Sciences, University of Bristol, Woodland Road, Bristol, BS8 1UG, UK. ²Imaging Lab, Department of Archaeology and Anthropology, University of Bristol, 43 Woodland Road, Bristol, BS8 1UG, UK.

*To whom correspondence should be addressed. E-mail: fmontealegrez@lincoln.ac.uk (F.M.-Z.); d.robert@bristol.ac.uk (D.R.)

†Present address: School of Life Sciences, Riseholme Campus, University of Lincoln, Lincoln, LN2 2LG, UK.

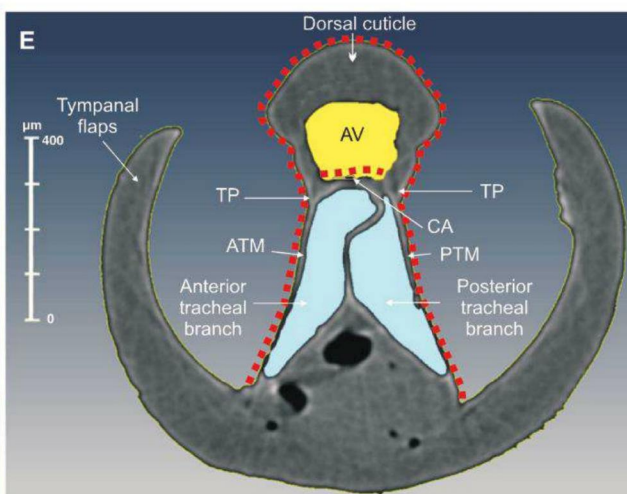
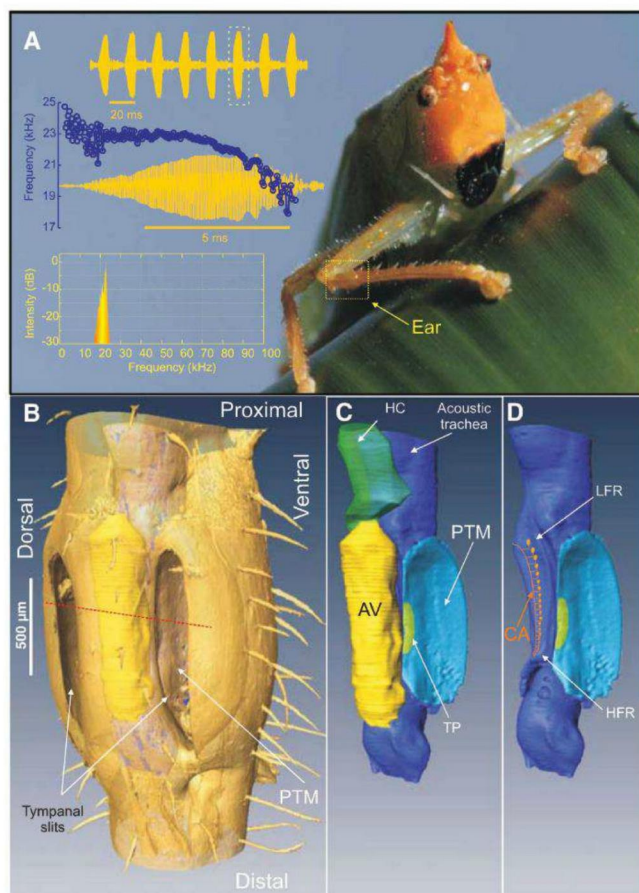


Fig. 2. Ears and song of *C. gorgonensis*. (A) The male calling song shown in time (oscillograms) and frequency domains. Blue data show the Hilbert transform of one song pulse (denoted by the dashed rectangle), revealing substantial frequency modulation (23.5 to 18.2 kHz). (B) X-ray μ CT reconstruction of female proximal tibia and external auditory morphology (see fig. S1, for male, and movie S1). The red dashed line denotes the axis of the cross section shown in (E). (C) μ CT reconstruction of internal organization showing the acoustic trachea (dark blue), PTM (light blue), TP (light green), fluid-filled AV (yellow), and HC (dark green). (D) Digital removal of the AV allows visualization of the mechanosensory organ, the CA with 28 scolopidia, and the size-gradient from distal high-frequency to proximal low-frequency receptors (HFR and LFR, respectively). (E) Internal auditory anatomy shown in a cross-sectional view: ATM and PTM, air-filled tracheal branches (blue), and the AV. The CA is wedged between the AV and the dorsal wall of the anterior tracheal branch. Red dotted lines indicate the location of LDV measurements.

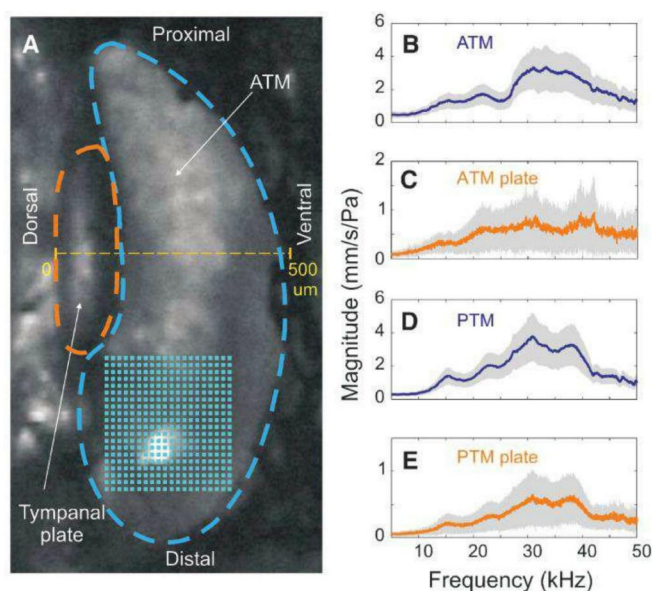
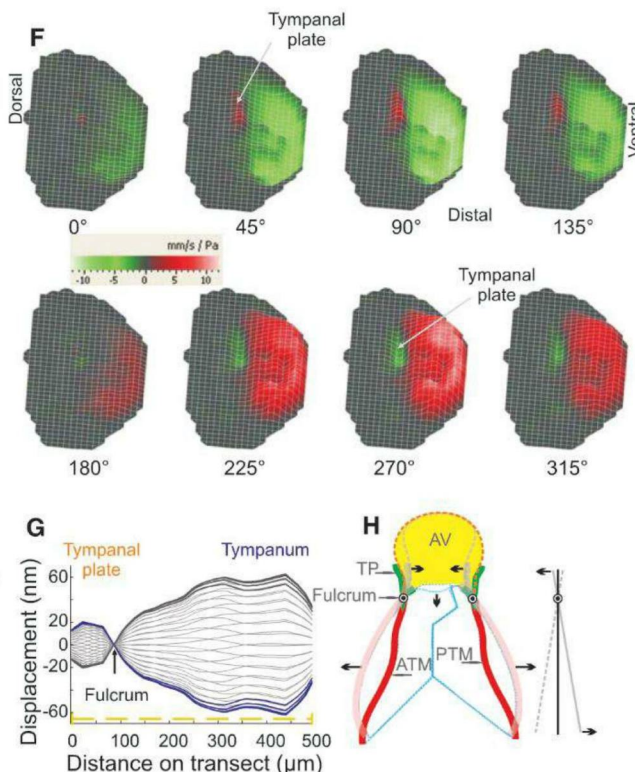


Fig. 3. Impedance conversion by tympanal mechanics. (A) Layout of the external ear as monitored by LDV. The blue dashed outline delineates the ATM; the orange dashed outline shows the associated ATM plate (ATP). The blue lattice illustrates the density of LDV measurements of ATM and ATP vibrations. (B to E) Average velocity spectra from one animal. (B) ATM (± 1 SD, $n = 1500$ scan points); (C) ATP (± 1 SD, $n = 330$ points); (D) PTM; (E) PTM plate (PTP). (F) Deflection maps of mechanical responses of ATM and ATP to the 23-kHz tone. Red and green indicate outward and inward deflections, respectively, shown every 45° through one oscillation cycle. (G) Deflection envelope of ATM and ATP [across the yellow dashed line in (A)], shown every 10° along the oscillation cycle. ATM and ATP oscillations are exactly out of phase with each other. (H) Model of impedance converter, using schematic cross section (Fig. 2E) and lever type 1 analogy.



analytical acoustic stimuli (Fig. 3A). All ear inputs (tympana and spiracles) were exposed to equal sound pressures, using either broadband sounds (5 to 50 kHz) or four-cycle tones at various frequencies, including the 23-kHz calling song (14). The auditory frequency range of both TMs exhibits a broad mechanical tuning between 15 and 40 kHz, with a series of maxima in mechanical sensitivity ranging from 20 to 30 kHz (for instance, at 15, 25, 30, and 40 kHz; $n = 21$ animals) (Fig. 3, B and D, and fig. S3).

Both anterior and posterior tympana undergo identical modes of vibration with maximum deflection magnitudes in ventral regions (Fig. 3F), as previously reported in other katydid species (8, 15). From ventral to dorsal side, the TM (Fig. 3A, blue dashed outline) vibrates with steadily decreasing amplitude (Fig. 3F). Extending LDV analysis past the dorsal edge of the TM reveals the vibrations of a cuticular patch, the tympanal plate (TP) (Fig. 3A, orange dashed outline), which makes tight contact with the distal end of the AV. TM and TP vibrations are spectrally similar (Fig.

3, B and E, and fig. S3), indicating that frequency decomposition does not take place at this early stage in the chain of hearing.

Importantly, TM and TP vibrations are 180° out of phase, as illustrated by deflection maps and profiles (Fig. 3G and movie S2). Together, deflection patterns and phase response show that the TM and TP rock around a stationary fulcrum and show rigid connection (Fig. 3G). This rocking behavior is observed for four-cycle tones at any frequency (5 to 50 kHz) and broadband sweeps (fig. S3).

This morphology and mechanical response reveals that the TM-TP system (14) acts as an impedance converter analogous to the middle ear ossicles described in terrestrial tetrapods (16, 17). The large deflections of the airborne TM are transformed into smaller deflections of the fluid-bound TP. Thus, this lever system converts a large displacement generated by a small force over a large surface area (sound pressure) (TM) into a small displacement imparting a larger force over a smaller surface area (TP), which is then coupled to AV.

From the linear proportions between the TM and TP, the lever ratio is $\sim 10:1$ (Fig. 3, G and H). In addition, the ratio between the TM and TP surface areas is $12.94 (\pm 1.12, n = 21)$ animals). The TM-TP system thus operates like a type 1 lever, with at least a 1:10 conversion between effort and load (Fig. 3H). Although TM and TP surface areas are significantly larger in females than in males, male and female TM:TP ratios do not differ (table S1). Amplifying the force imparted by the TMs to the AV and CA, this lever ratio is similar to that reported for the coupling of the tympanal membrane to the cochlea's oval window via the middle ear ossicles in mammals (range: 12.5 to 21.1) (16, 18).

The dorsal cuticle covering the AV between the anterior and posterior tympanal membranes (ATM and PTM, respectively) (Fig. 2E) exhibits a mechanical sensitivity to sound (ratio between response velocity and sound pressure level; millimeters per second per pascal) as high as that observed for the TM and TP (Fig. 4A). Mechanically, this response is representative of AV

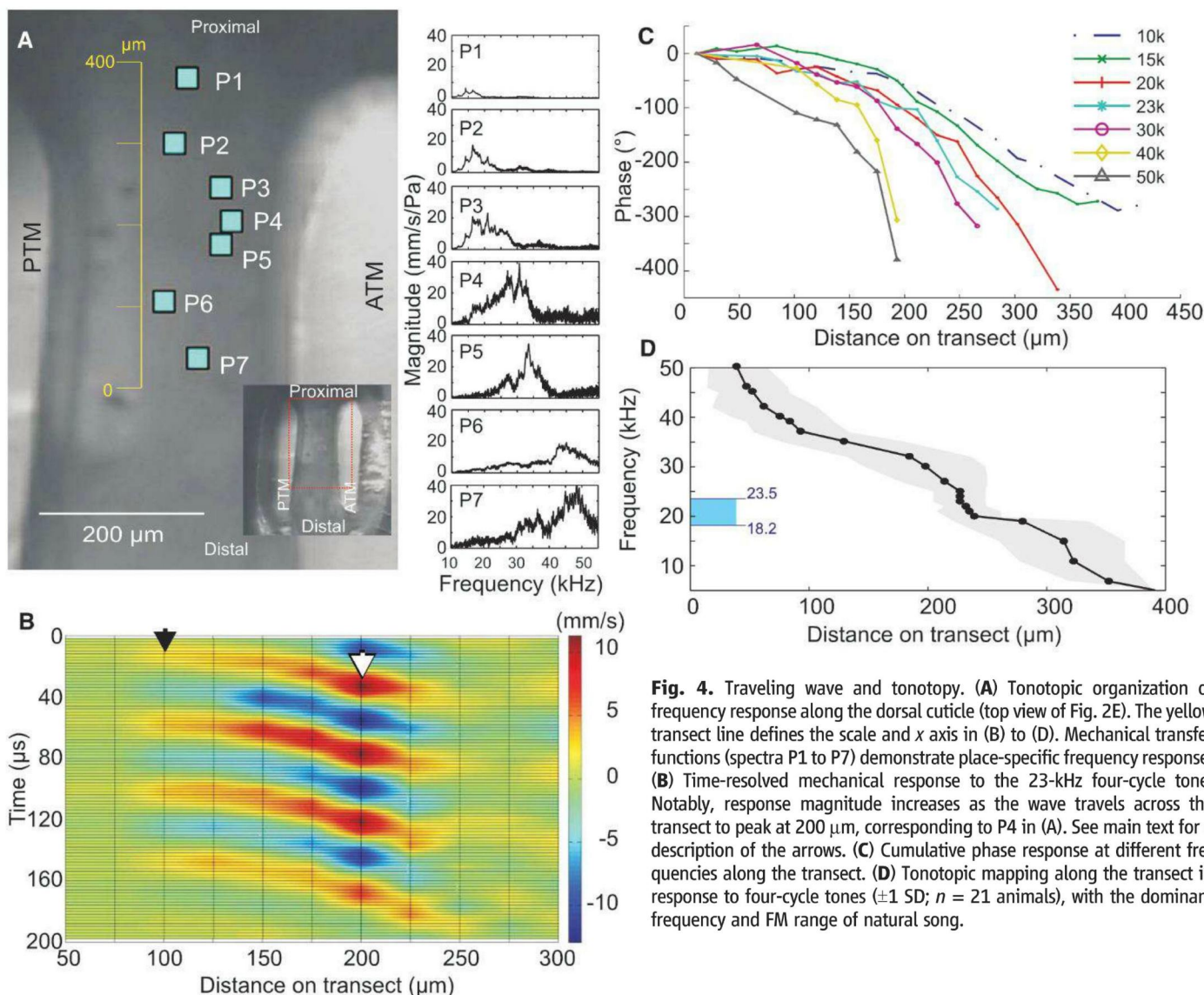


Fig. 4. Traveling wave and tonotopy. (A) Tonotopic organization of frequency response along the dorsal cuticle (top view of Fig. 2E). The yellow transect line defines the scale and x axis in (B) to (D). Mechanical transfer functions (spectra P1 to P7) demonstrate place-specific frequency response. (B) Time-resolved mechanical response to the 23-kHz four-cycle tone. Notably, response magnitude increases as the wave travels across the transect to peak at 200 μm, corresponding to P4 in (A). See main text for a description of the arrows. (C) Cumulative phase response at different frequencies along the transect. (D) Tonotopic mapping along the transect in response to four-cycle tones (± 1 SD; $n = 21$ animals), with the dominant frequency and FM range of natural song.

vibrations, as established by other experiments (figs. S4 to S6) (14).

The spatial organization of the frequency response along the dorsal cuticle is in stark contrast with that of the TM and TP. Low frequencies dominate at the proximal end (P1 in Fig. 4A), whereas high frequencies prevail distally (P7 in Fig. 4A; see also fig. S5). This is a hallmark of tonotopy. This organization conforms to the tonotopic sensitivities of CA mechanoreceptors (Fig. 2D) (11). Intermediate locations (P2 to P6) exhibit a gradual increase in frequency response.

The definitive characteristics of mammalian cochlear-frequency analyzers are the essential unidirectional traveling waves, discovered by Georg von Békésy (3). These features are paralleled at the microscale in katydid by the mechanical deflections observed along the dorsal cuticle (Fig. 4A, yellow line). Traveling waves are initiated distally and travel toward proximal locations of the AV, as recorded through the dorsal cuticle (Fig. 4B and movie S3). A 23-kHz four-cycle tone elicits four oscillations that all reach an identical physical destination, located 200 μm along the transect around location P3-P4 (Fig. 4, A and B). The first cycle elicits a sinusoidal response first located at $\sim 100 \mu\text{m}$ (Fig. 4B, black arrow), which then moves toward the 200- μm mark (Fig. 4B, white arrow) within 22 μs (half-period at 23 kHz). The oscillation reaches its maximum displacement velocity (red and blue peaks in Fig. 4B) at this position (200 μm), before rapidly decaying over a further 25 μm . The next three cycles generate the same oscillations with increased magnitude at this location. The presence of a wave traveling from distal to proximal locations along the CA corroborates results from another katydid species (13). Other frequencies (5 to 50 kHz) also elicit this pattern but reach maximal magnitude at different locations (Fig. 4D). The dispersive nature of AV waves is best illustrated by the presence of phase accumulation, as waves travel across the dorsal cuticle. High-frequency waves (40 to 50 kHz) incur some 260° phase lag within a 200- μm travel length, whereas low-frequency waves (10 kHz), traveling 400 μm , accumulate up to 300° (Fig. 4C). The velocity of wave propagation varies with frequency from 4 ms^{-1} (10 kHz) to 8 ms^{-1} (50 kHz), exhibiting a pattern similar to those reported for mammals and other insects (13, 19).

Deflection shape analysis conducted in the spectral domain (5- to 50-kHz sweeps) confirms the unambiguous presence of traveling waves (fig. S6) (14). The data imply that AV provides the anisotropic medium enabling dispersive wave propagation and tonotopic delivery to the auditory receptors. Further experiments establish that both an intact AV and its fluid are required for frequency decomposition as well as for generation of traveling waves (fig. S7). In contrast to observations from the phaneropterine katydid *Mecopoda elongata* (13), removal of the dorsal cuticle in *C. gorgonensis* markedly alters AV

integrity and eliminates traveling waves and dispersive propagation (14).

Altogether, the data show that the impedance conversion, dispersive wave propagation, and tonotopic representation are biophysically analogous to the same qualities of the mammalian cochlea. For *C. gorgonensis*, however, the entire process is embedded in morphology about one to two orders of magnitude smaller than that of mammals (centimeters and millimeters to micrometers).

Frequency representation along the AV covers 10 to 50 kHz (Fig. 4D), a range much larger than that of the natural song (FM: 23.5 to 18.2 kHz) (Fig. 2A) (6). Notably, only a short AV segment of $\sim 40 \mu\text{m}$ represents the song's FM ($n = 21$ animals) (Fig. 4D). For other frequencies, tonotopic mapping is spatially more disperse (Fig. 4D and fig. S6), suggesting that *C. gorgonensis* can hear other sounds in addition to its own song, such as the ultrasonic sounds of predators (e.g., echolocating bats) (20).

As in mammals, impedance matching in katydids arises from the surface area ratio between the TM and TP and from the lever resulting from their coupled action (Fig. 1). Both cases are evolutionarily convergent; the result is the efficient transfer of vibrations to the fluid- or lipid-filled channel (the cochlea or the AV, respectively) where mechanosensory cells reside. Sophisticated hearing is possible at the microscale; katydid ears provide valuable inspiration for the construction of miniaturized smart acoustic sensors, contributing to the expanding panoply of insect-inspired technology.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/338/6109/968/DC1

Materials and Methods

Supplementary Text

Figs. S1 to S7

Table S1

References (21–32)

Movies S1 to S3

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Offspring from Oocytes Derived from in Vitro Primordial Germ Cell-like Cells in Mice

Katsuhiko Hayashi,^{1,2,3*} Sugako Ogushi,^{1,4} Kazuki Kurimoto,^{1,5} So Shimamoto,¹ Hiroshi Ohta,^{1,5} Mitinori Saitou^{1,2,5,6*}

Reconstitution of female germ cell development in vitro is a key challenge in reproductive biology and medicine. We show here that female (XX) embryonic stem cells and induced pluripotent stem cells in mice are induced into primordial germ cell-like cells (PGCLCs), which, when aggregated with female gonadal somatic cells as reconstituted ovaries, undergo X-reactivation, imprint erasure, and cyst formation, and exhibit meiotic potential. Upon transplantation under mouse ovarian bursa, PGCLCs in the reconstituted ovaries mature into germinal vesicle-stage oocytes, which then contribute to fertile offspring after in vitro maturation and fertilization. Our culture system serves as a robust foundation for the investigation of key properties of female germ cells, including the acquisition of totipotency, and for the reconstitution of whole female germ cell development in vitro.

The germ cell lineage in mammals originates from pluripotent epiblasts as primordial germ cells (PGCs) and undergoes sexually

dimorphic development, generating spermatozoa in males and oocytes in females. These cells fertilize to form zygotes with full developmental

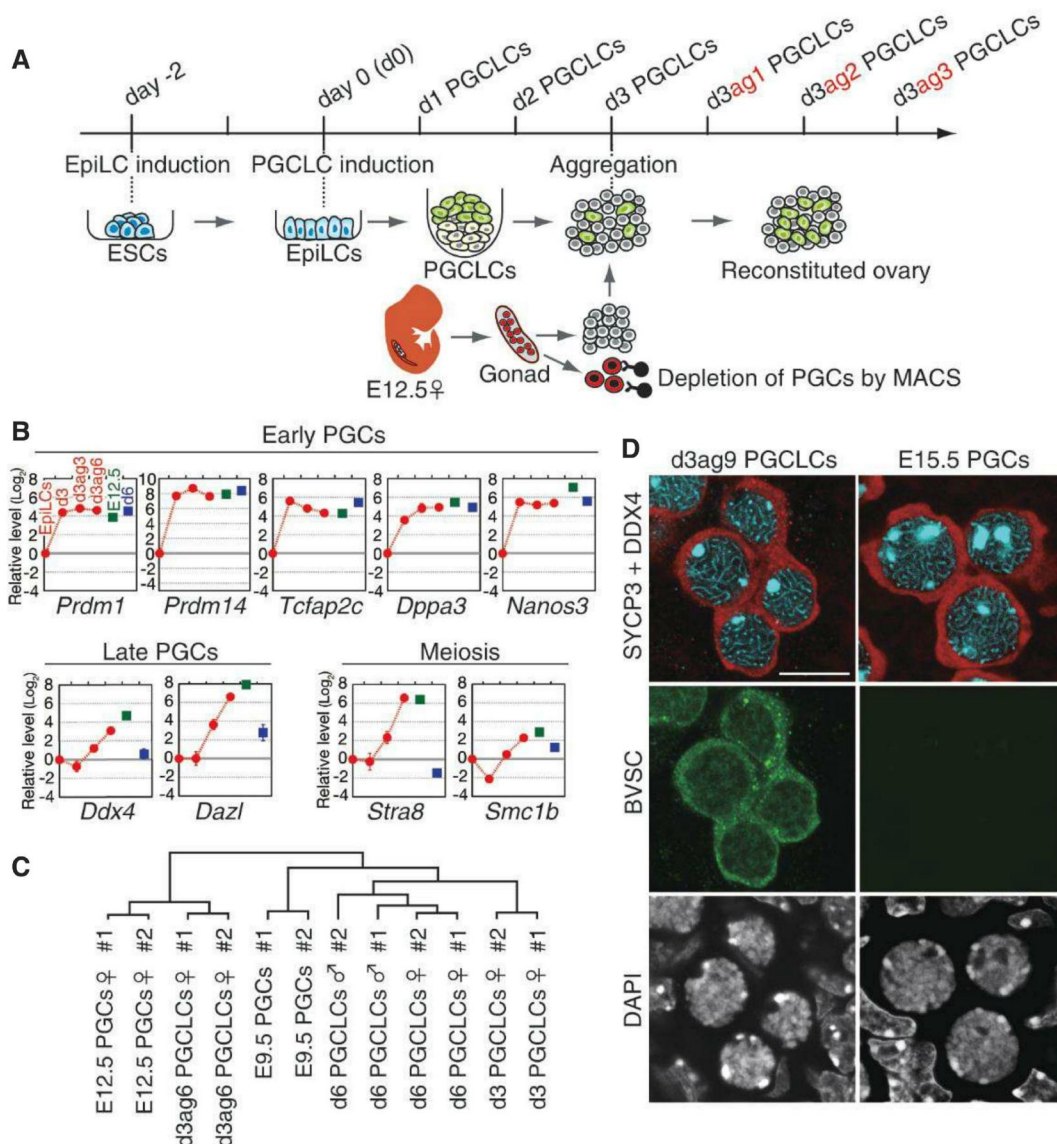
potential, serving as the foundation of the mammalian life cycle. We have shown previously that embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) from male (XY) mice are induced into epiblast-like cells (EpiLCs), which,

in turn, are induced into primordial germ cell-like cells (PGCLCs) with proper function for spermatogenesis and offspring production (1). Considering the differences between male (XY) and female (XX) ESCs, including those at the epigenetic level (2), together with the markedly different mechanisms of male and female germ cell development (3), a fundamental challenge remains to determine whether our system reconstitutes the female developmental pathway and whether PGCLCs serve as precursors for fully functional oocytes.

To explore this possibility, we derived female ESCs bearing *Prdm1* (also known as *Blimp1*)–*mVenus* and *Dppa3* (also known as *stella*)–*ECFP* (BVSC) transgenes (*Prdm1* and *Dppa3* show specific expression in PGCs) (4) and induced them into EpiLCs and further into PGCLCs (see supplementary materials and methods). The dynamics of EpiLC and PGCLC induction from the female ESCs (the H18 and H14 lines) appeared similar to those from male ESCs (fig. S1) (1). To

further characterize female PGCLCs, we chose the strategy of “reconstituted ovaries”: One can aggregate female PGCs with embryonic gonadal somatic cells to form reconstituted ovaries in vitro, which are then transplanted under ovarian bursa or kidney capsules of recipient mice for oogenesis (Fig. 1A) (5, 6). The H18 ESCs were induced into PGCLCs, and, at each time point from day 3 (d3) to day 6 (d6) of PGCLC induction, the BV-positive cells were sorted by fluorescence-activated cell sorting and reaggregated with E12.5 (embryonic day 12.5) gonadal somatic cells (1000 PGCLCs plus 10,000 somatic cells) for further culture (Fig. 1A and fig. S2A). The d3 PGCLCs exhibit robust proliferation in reconstituted ovaries (fig. S2 and supplementary text) and, with regard to gene expression and epigenetic profiles (X inactivation or reactivation and imprinting), undergo a developmental progression similar to that of PGCs in embryonic ovaries, reaching, at day 6 in reconstituted ovaries (d3ag6), a premeiotic stage similar to E12.5 PGCs (Fig. 1, B

Fig. 1. Female PGCLC development in reconstituted ovaries. **(A)** Scheme for PGCLC induction and ovary reconstitution with d3 PGCLCs. See also fig. S2A. MACS, Magnetic-activated cell sorting. **(B)** Gene expression during PGCLC development and in E12.5 PGCs. For each gene, we calculated the ΔCT (the difference in the threshold cycles) from the average CT values of the two independent housekeeping genes *Arbp* and *Ppia*. The value for EpiLCs was set as 0. As represented in the *Prdm1* panel (upper left), for each point, the average value from two independent experiments is shown on the log₂ scale (with standard deviations) for d3 PGCLC development (EpiLCs, d3, d3ag3, d3ag6 PGCLCs, red circles), E12.5 PGCs (green squares), and d6 PGCLCs (blue squares). **(C)** Hierarchical clustering of microarray data for d3 (female), d6 (male and females), and d3ag6 (female) PGCLCs, as well as E9.5 (mixed) and E12.5 (female) PGCs. **(D)** Expression of DDX4 (DEAD box polypeptide 4, a cytoplasmic RNA helicase expressed in germ cells after ~E11.5) (red), SYCP3 (synaptonemal complex protein 3, a component of the synaptonemal complex between meiotic homologous chromosomes, light blue), and the BVSC (*Blimp1-mVenus* and *stella-ECFP*) reporter (green) and 4',6-diamidino-2-phenylindole (DAPI) staining (white) of d3ag9 PGCLCs (d3 PGCLCs cultured in the reconstituted ovary for 9 days) and E15.5 PGCs. Scale bar, 10 μ m.



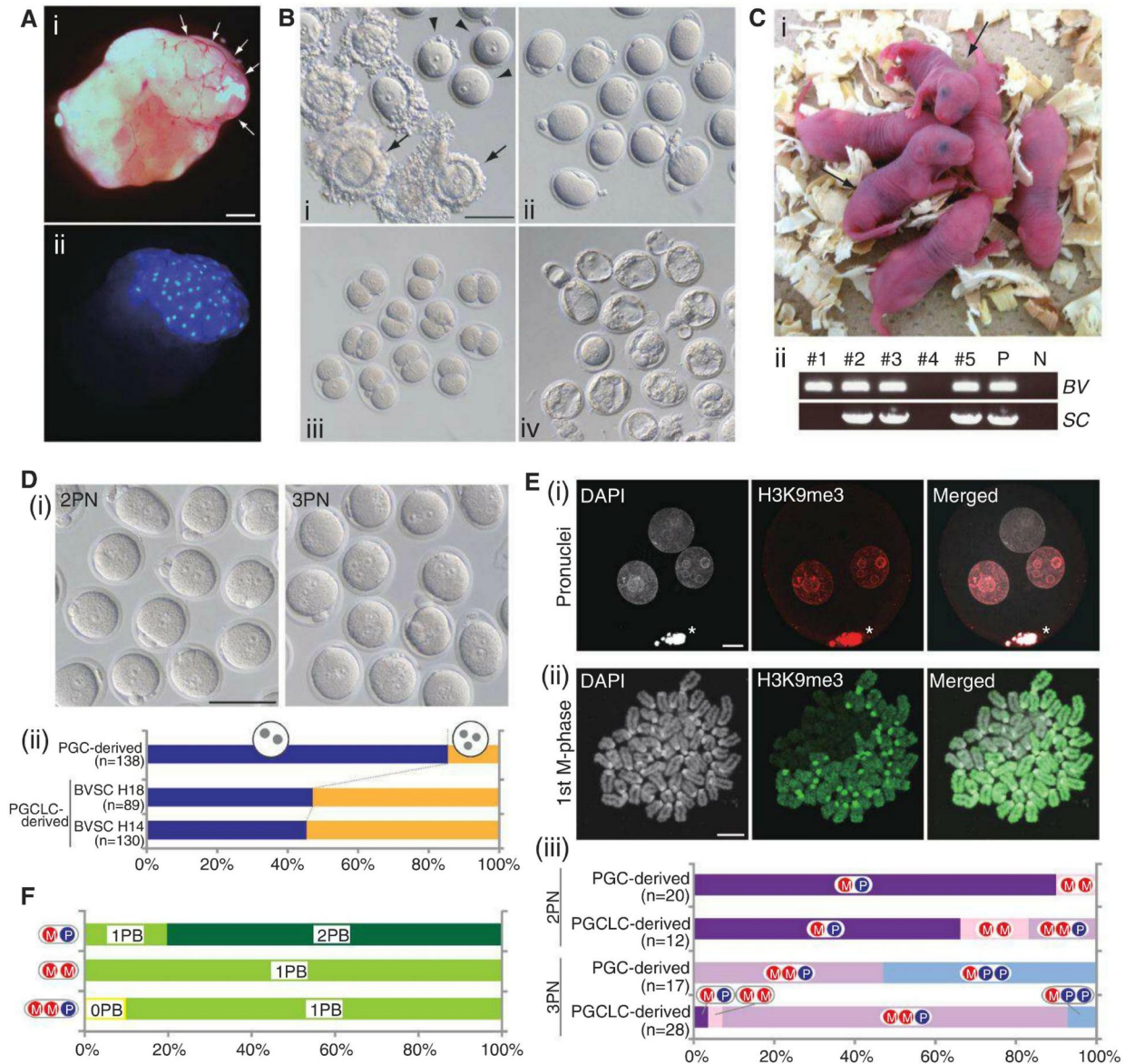


Fig. 2. Oogenesis, embryonic development, and offspring from female PGCLCs. **(A)** Oogenesis by PGCLCs in reconstituted ovaries. (i) Bright-field image of the recipient and reconstituted (arrows) ovary complex. Scale bar, 500 μ m. (ii) CFP (cyan fluorescent protein) fluorescence of the complex. **(B)** Maturation by IVM and in vitro development after IVF of PGCLC-derived oocytes. Bright-field images for GV (i) and MII (ii) oocytes, two-cell embryos (iii), and blastocysts (iv). Arrows denote COCs; arrowheads indicate DOs. Scale bar, 100 μ m. **(C)** (i) PGCLC-derived offspring (black-eyed, arrow) and (ii) their genotyping by the BVSC transgenes with positive (P) and negative (N) controls. **(D)** (i) PGCLC-derived zygotes and oocytes after IVF bearing 2PN (left) or 3PN

(right), judged by visual inspection. Scale bar, 100 μ m. (ii) Proportion of 2PN and 3PN zygotes and oocytes after IVF of oocytes from PGCs and PGCLCs (H18 and H14 ESC-derived). n, number of zygotes examined. **(E)** (i) A digynic triploid zygote with one (first) PB (*), stained for H3K9me3 (red) and DAPI (white). (ii) Chromosome spread at the first M phase of a digynic triploid zygote stained for H3K9me3 (green) and DAPI (white). Scale bars in (i) and (ii), 10 μ m. (iii) Proportion of the indicated chromosome constitutions in PGCLC- or PGC-derived zygotes and oocytes. M, maternal; P, paternal. **(F)** PBs observed in the PGCLC-derived zygotes. OPB, no PB detected; 1PB, only the first PB detected; 2PB, the second PB detected.

Table 1. Generation of offspring from oocytes derived from 3-week ovaries, E12.5 PGCs, and PGCLCs. —, not determined; b, black-eyed; al, albino.

Donor	Transplants recovered	Total GVs (/transplant)	MI I oocytes (%)	IVF	Fertilized (%)	Culture	Two-cell embryos (%)	Embryos transferred	Pups (%)
Oocytes (3 weeks)	—	165	65 (39.4)	59	57 (96.6)	57	55 (96.5)	55	7 (12.7)
E12.5 PGCs (SG)	15	485 (32.3)	223 (46.0)	223	127 (57.0)	113	85 (75.2)	75	13 (17.3)
d3 PGCLCs (ESCs)	16	535 (33.4)	319 (59.6)	243	204 (84.0)	204	127 (62.3)	127	5 (3.9)
d6 PGCLCs (ESCs)	20	62 (3.1)	28 (45.2)	—	—	—	—	—	—
d4 PGCLCs (iPSCs)	36	1565 (43.5)	507 (32.4)	507	342 (67.5)	236	163 (69.1)	163	14 (b 3; al 11)
E12.5 PGCs*	10	145 (14.5)	67 (46.2)	—	—	—	—	—	—

*PGCs remaining to be sorted out by MACS.

and C, figs. S3 and S4, and supplementary text). We validated that in reconstituted ovaries, d3 and d6 PGCLCs can progress into the zygotene stage of the meiotic prophase [judged by the expression and localization pattern of SYCP3 (synaptonemal complex protein 3) (Fig. 1D, fig. S3, and supplementary text)], demonstrating the meiotic potential of PGCLCs.

To explore the potential of PGCLCs for oogenesis, we generated the reconstituted ovaries with d3 PGCLCs, d6 PGCLCs, and stella-EGFP (SG)-positive E12.5 female PGCs (7) (5000 PGCLCs or E12.5 PGCs plus 50,000 somatic cells), respectively, and transplanted them at d2 of culture under the ovarian bursa of nude mice (two reconstituted ovaries per recipient ovary). Four weeks and 4 days after transplantation, we isolated the complex of the recipient and reconstituted ovaries. The reconstituted ovaries with PGCLCs, especially those with d3 PGCLCs, exhibited substantial growth, were well vascularized, and exhibited strong SC [*Dppa3/stella-EGFP*] fluorescence, which indicates the presence of growing or grown oocytes (Fig. 2A and fig. S5A). Histological sections revealed that the SC-positive donor cells contribute to oocyte-like cells, and nearly all of them reached the fully grown germinal vesicle (GV) stage, bearing multiple layers of granulosa and theca cells similar to the fully grown recipient follicles (fig. S5B). We found no primordial or primary donor-derived follicles (fig. S5, A and B), indicating that PGCLCs underwent oogenesis in a synchronous single wave. Similarly, E12.5 PGCs underwent oogenesis in reconstituted ovaries in a single wave (fig. S5A), which is consistent with a previous observation (6).

To evaluate the morphology and efficiency of the formation of GV oocytes by PGCLCs and PGCs, we mechanically isolated follicles and oocytes in the reconstituted ovaries. We also mechanically isolated GV follicles and oocytes in wild-type (WT) ovaries at 3 weeks. The average numbers of d3 PGCLC- and E12.5 PGC-derived GV oocytes per reconstituted ovary were comparable (33.4 and 32.3, respectively), whereas that of d6 PGCLCs was smaller (3.1) (Table 1). This may be partly because d3, but not d6 PGCLCs proliferate robustly in reconstituted ovaries (fig. S2). Whereas most (~65%) of the WT oocytes at 3 weeks were isolated as a cumulus cell-oocyte complex (COC), most (~60%) of the oocytes derived from PGCLCs and PGCs were isolated as denuded oocytes (DOs) (fig. S6A). Moreover, the oocytes derived from the PGCLCs exhibited abnormal elliptical shape at a higher frequency than the oocytes of the other two groups (fig. S6A). These results suggest that, although the development of the PGCLC- and E12.5 PGC-reconstituted ovaries appears grossly normal, it may involve some instability in COC formation and that the GV oocytes from PGCLCs may exhibit some cytoskeletal immaturity and/or fragility with a certain frequency.

We performed in vitro maturation (IVM) and in vitro fertilization (IVF) of the d3 PGCLC- and

PGC-derived oocytes and the WT oocytes at 3 weeks (fig. S6B). Despite differences in COC stability and shape, the PGCLC-derived oocytes reached metaphase II (MII), were fertilized, and developed into two-cell embryos with an efficiency comparable to that of oocytes from other sources (Fig. 2B, fig. S6C, and Table 1). Some of the two-cell embryos from the PGCLCs developed further into blastocysts in vitro [19 of 46 (19/46), ~39%] (Fig. 2B). We transferred the two-cell embryos from PGCLCs, as well as those from the other sources, to separate foster mothers. We obtained newborn pups from the two-cell embryos derived from PGCLCs (5/127, ~3.9%), as well as from those derived from E12.5 PGCs (13/75, ~17.3%) and WT 3-week oocytes (7/55, ~12.7%) (Fig. 2C, fig. S7A, and Table 1). All of these offspring grew similarly into adulthood (fig. S7C). The PGCLC-derived offspring bore the BVSC transgenes, a normal imprinting pat-

tern, and full fertility (Fig. 2C and fig. S7, B and D). These findings demonstrate that female PGCLCs induced from ESCs in vitro can form fully functional oocytes.

Nonetheless, it was less efficient to obtain PGCLC-derived pups (~3.9%) than E12.5 PGC- or WT 3-week oocyte-derived pups (~12.7 and ~17.3%, respectively) (Table 1). To gain insight into the underlying mechanisms, we examined the meiosis of the oocytes from PGCLCs. Most PGCLC-derived MII-oocytes induced by IVM bore apparently normal meiotic spindles and the first polar bodies (PBs) (fig. S8A), indicating relatively normal first meiotic division of the PGCLC-derived oocytes. However, when we scrutinized the PGCLC-derived zygotes formed by IVF at the pronuclear stage, we found that about half (~52.8%) of them had three pronuclei (3PN), whereas a majority (~85.5%) of PGC-derived oocytes had a normal two-pronuclear

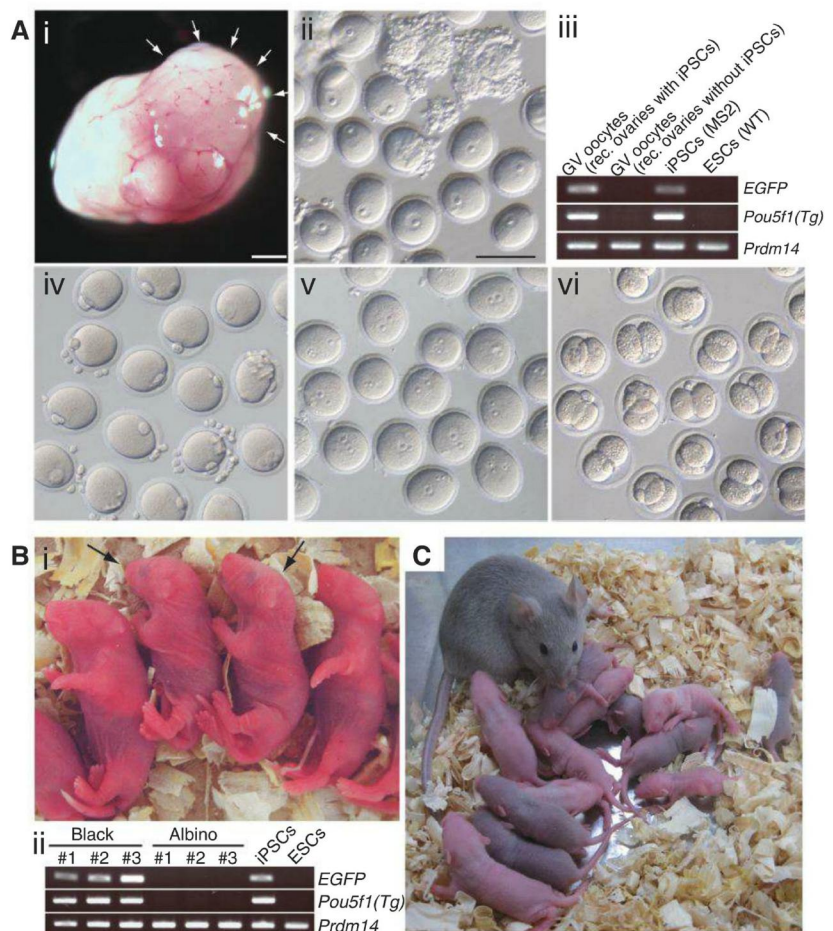


Fig. 3. Oogenesis, embryonic development, and offspring from female iPSC-derived PGCLCs. **(A)** Oogenesis and embryonic development from iPSC-derived PGCLCs. (i) Bright-field image of the recipient and reconstituted (arrows) ovary complex. Scale bar, 500 μ m. (ii) GV oocytes isolated from reconstituted ovaries. Scale bar, 100 μ m. (iii) Genotyping by indicated elements of GV oocytes from reconstituted ovaries with iPSC-derived PGCLCs or from control ovaries, iPSCs, and WT ESCs. (iv to vi) MII oocytes (iv), zygotes (v), and two-cell embryos (vi) from the GV oocytes in (ii). **(B)** (i) Black-eyed offspring from iPSC-derived PGCLCs (arrows) and (ii) their genotyping by indicated elements, with genotyping of albino offspring, iPSCs, and WT ESCs. **(C)** Fertile female offspring from iPSC-derived PGCLCs.

(2PN) phenotype (Fig. 2D). The H14 ESC-derived PGCLC-derived zygotes showed a similar phenotype (Fig. 2D), indicating that the emergence of zygotes and oocytes bearing 3PN after IVF at a higher ratio is a feature of oocytes derived from female ESCs. Most PGCLC-derived 3PN zygotes had two maternal (M) chromosomes positive for histone H3 lysine nine trimethylation (H3K9me3) (8) and one paternal (P) chromosome, whereas about ~65% of the 2PN zygotes from PGCLCs had one M and one P chromosome and the rest had either two Ms or two Ms and one P (Fig. 2E). All of the 3PN zygotes examined (10/10) failed to extrude the second PBs and carried the triploid chromosomes into two-cell embryos (Fig. 2F and fig. S8, B and C). These findings indicate that a subset of the zygotes and oocytes derived from PGCLCs are unable to extrude the second PBs, resulting, after IVF, in the digynic triploid (MMP) phenotype or the digynic diploid (MM) phenotype with a failure in fertilization. This defect contributes to a low birth rate from the two-cell embryos derived from PGCLCs (Table 1). Consistent with these findings and the fact that the triploidy is incompatible with mouse development (9), all of the offspring from PGCLC-derived oocytes exhibited a normal diploid karyotype (fig. S8D).

We examined whether female iPSCs could similarly be induced into fully functional oocytes. We induced the MS2 iPSCs (derived from female mouse embryonic fibroblasts, mixed background with C57BL/6, and bearing *Pou5fl-EGFP* transgenes) (10) into PGCLCs. We purified integrin- β 3- and SSEA1-positive cells, which are nearly identical to cells with BV positivity (1), from the floating aggregates containing d4 PGCLCs (we used d4 PGCLCs because integrin- β 3 exhibits specific expression after d4 of PGCLC induction) (fig. S9, A and B) and generated reconstituted ovaries. As a control, we generated reconstituted ovaries without the iPSC-derived PGCLCs, but with a small number of unsorted endogenous PGCs (ICR background). After transplantation, the reconstituted ovaries with the iPSC-derived PGCLCs bore many more growing or grown follicles (43.5 per transplant) than the controls (14.5 per transplant) (Fig. 3A and Table 1), suggesting that the iPSC-derived PGCLCs contributed successfully to oogenesis. Although the *Pou5fl-EGFP* transgenes did not show detectable fluorescence in the reconstituted ovaries (fig. S9, C to F), the GV oocytes from reconstituted ovaries containing the iPSC-derived PGCLCs specifically bore the *EGFP* and *Pou5fl* retrovirus transgenes, as did the original MS2 iPSCs (Fig. 3A), demonstrating the contribution of the iPSC-derived PGCLCs to oogenesis. We performed IVM and IVF using these oocytes and transferred two-cell embryos derived from zygotes with 2PN to foster mothers (Fig. 3A). We obtained three offspring with black eyes, which bore the *EGFP* and the *Pou5fl* transgenes and grew into fertile adults (Fig. 3, B and C, and fig. S9G). We conclude that like female ESCs, female iPSCs can be induced into PGCLCs in vitro,

which, in turn, can mature into properly functioning oocytes.

A previous study showed that ESCs could differentiate into oocyte-like cells in culture, although these cells did not contribute to offspring (11). We demonstrate here that female ESCs and iPSCs were induced into PGCLCs, which underwent proper development in reconstituted ovaries in vitro and matured further into fully functional GV oocytes upon transplantation in vivo. Combined with our previous study (1), reconstitution of the essential first step of pluripotent stem cell-based gamete production in vitro has been established in both sexes. Unlike the E12.5 PGC-derived oocytes and zygotes, about half of the PGCLC-derived oocytes and zygotes failed to extrude the second PBs. The underlying mechanism of this failure requires further investigation. Combined with previous studies (12, 13), our system serves as a robust foundation to investigate and further reconstitute female germline development in vitro, not only in mice, but also in other mammals, including humans.

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Supplementary Materials

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A Genomic Regulatory Element That Directs Assembly and Function of Immune-Specific AP-1–IRF Complexes

Elke Glasmacher,^{1*} Smita Agrawal,^{1*} Abraham B. Chang,^{1*} Theresa L. Murphy,^{2*} Wenwen Zeng,^{1*} Bryan Vander Lugt,¹ Aly A. Khan,³ Maria Ciofani,⁴ Chauncey J. Spooner,¹ Sascha Rutz,¹ Jason Hackney,⁵ Roza Nurieva,⁶ Carlos R. Escalante,⁷ Wenjun Ouyang,¹ Dan R. Littman,⁴ Kenneth M. Murphy,^{2†} Harinder Singh^{1†}

Interferon regulatory factor 4 (IRF4) and IRF8 regulate B, T, macrophage, and dendritic cell differentiation. They are recruited to cis-regulatory Ets-IRF composite elements by PU.1 or Spi-B. How these IRFs target genes in most T cells is enigmatic given the absence of specific Ets partners. Chromatin immunoprecipitation sequencing in T helper 17 (T_H17) cells reveals that IRF4 targets sequences enriched for activating protein 1 (AP-1)–IRF composite elements (AICEs) that are co-bound by BATF, an AP-1 factor required for T_H17, B, and dendritic cell differentiation. IRF4 and BATF bind cooperatively to structurally divergent AICEs to promote gene activation and T_H17 differentiation. The AICE motif directs assembly of IRF4 or IRF8 with BATF heterodimers and is also used in T_H2, B, and dendritic cells. This genomic regulatory element and cognate factors appear to have evolved to integrate diverse immunomodulatory signals.

Interferon regulatory factor 4 (IRF4) and IRF8 are evolutionarily diverged members of the IRF family of transcription factors (1). Unlike other members, which are ubiquitously expressed, IRF4 and IRF8 are largely restricted to

the immune system and play key roles in the differentiation and functioning of innate and adaptive immune cells (2, 3). IRF4 is required for B cells to undergo class switch recombination and plasma cell differentiation (4, 5). It

additionally regulates the generation and/or functioning of various types of helper T (T_H) cells, including T_H17 (6), T_H2 (7, 8), follicular T_H (9),

¹Department of Discovery Immunology, Genentech, Incorporated, South San Francisco, CA 94080, USA. ²Department of Pathology and Immunology, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110, USA. ³Institute for Systems and Genomics Biology and Department of Human Genetics, The University of Chicago, Chicago, IL 60637, USA. ⁴Skirball Institute of Biomolecular Medicine, Howard Hughes Medical Institute, New York University, New York, NY 10016, USA. ⁵Department of Bioinformatics and Computational Biology, Genentech, Incorporated, South San Francisco, CA 94080, USA. ⁶Department of Immunology, M. D. Anderson Cancer Center, Houston, TX 77054, USA. ⁷Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, Richmond, VA 23219, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: kmurphy@pathology.wustl.edu (K.M.M.); harindsi@gene.com (H.S.)

and induced regulatory T cells (10). A distinction from other members of the IRF family is that IRF4 and IRF8 bind with low affinity to interferon-stimulated response elements (ISREs) (11, 12) (supplementary text S1). IRF4 and IRF8 have evolved to interact with other transcription factors so as to facilitate their recruitment to distinct genomic regulatory elements. The Ets family transcription factors, PU.1 and Spi-B, that are expressed in B cells, macrophages, and dendritic cells represent interaction partners that are best characterized (13–15). They are able to recruit IRF4 or IRF8 to composite Ets-IRF elements (EICEs). Chromatin immunoprecipitation sequencing (ChIP-seq) analysis of IRF4 in interleukin 21 (IL-21)-stimulated T cells that do not express PU.1 or Spi-B has revealed co-binding with signal transducer and activator of transcription 3 (STAT3) (16). The mechanism un-

derlying co-targeting of IRF4 and STAT3 to genomic regions remains to be defined but does not appear to involve DNA-dependent cooperative binding. Thus, in T_H cells that do not express PU.1 or Spi-B, it remains to be determined how IRF4 is recruited to various genomic targets.

We used ChIP-seq analysis to identify the interaction partners for IRF4 in T cells, reasoning that the DNA binding site of such a transcription factor(s) should be juxtaposed with the IRF motif in a stereospecific manner. ChIP-seq with IRF4 in J558L B cells that express PU.1 was used to test the utility of this approach. As anticipated, the EICE motif occurred with highest frequency in the targeted regions (fig. S1A). In contrast, ChIP-seq with IRF4 that is induced during T_H17 cell differentiation (fig. S1B) (supplementary text S2) revealed two types of composite IRF-AP-1 motifs, with either a 4- or 0-bp spacing (Fig. 1A).

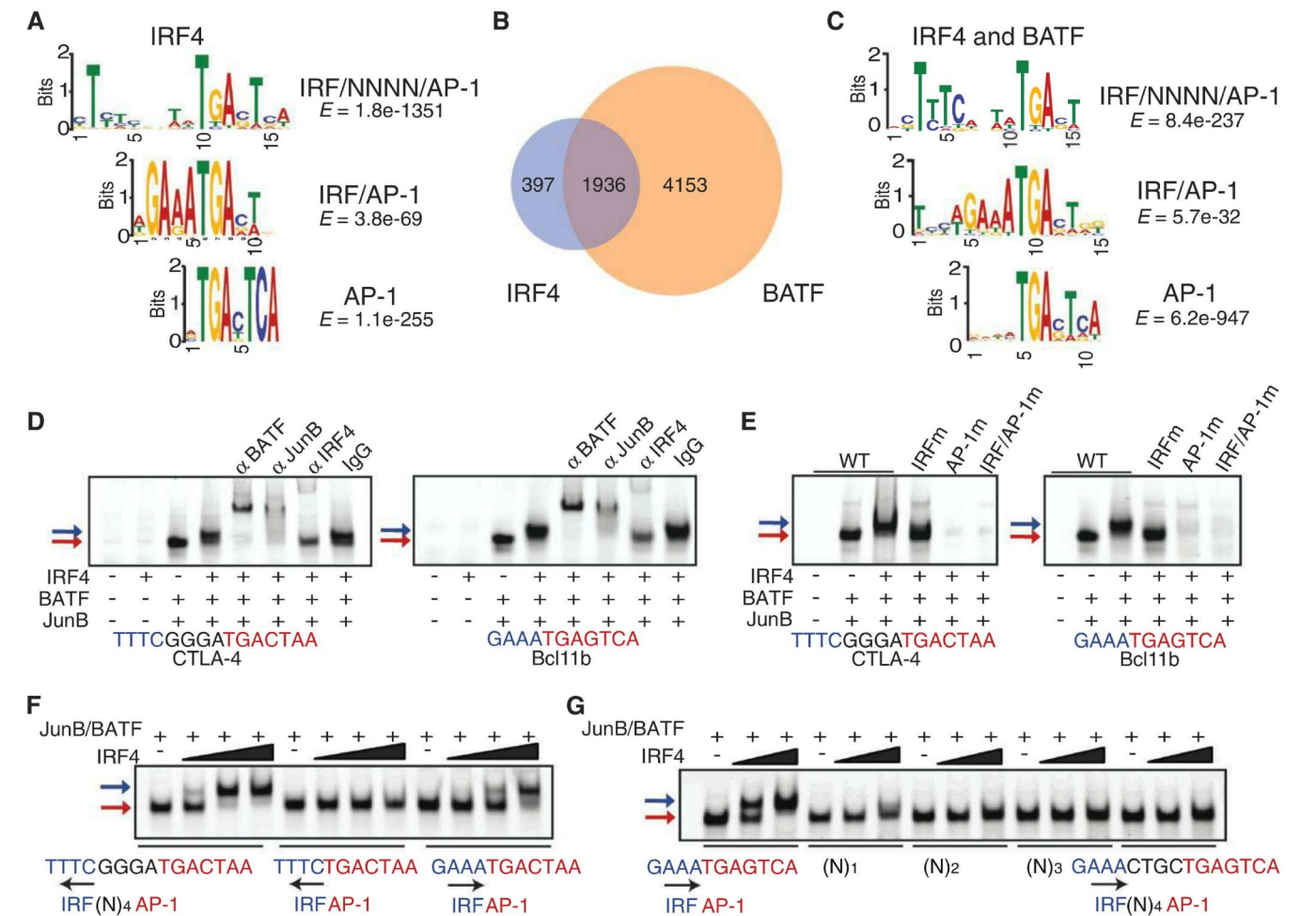


Fig. 1. IRF4 and BATF cistromes in T_H17 cells are enriched for composite AP-1-IRF motifs that direct cooperative binding. (A to C) ChIP-seq of in vitro differentiated T_H17 cells (42 hours) with antibodies directed against IRF4 or BATF. (A) MEME analysis of highly represented motifs within the IRF4 cistrome (2333 peaks) along with their statistical significance (*E*) values. (B) Union analysis of the IRF4 and BATF cistromes. Numbers indicate unique or coincident peaks, the latter with peak maxima within 100 bp of each other. (C) MEME analysis of highly represented motifs within the coincident peak sequences of IRF4 and BATF (1936 peaks). (D to G) EMSAs using nuclear extracts from 293T cells overexpressing IRF4, BATF, or JunB. The AP-1 (red) and IRF (blue) sites are

indicated. The sequences were derived from IRF4 and BATF co-targeted regions within the CTLA-4 or Bcl11b loci. (D) Protein-DNA complexes were supershifted with antibodies indicated above. (E to G) EMSAs using wild-type (WT) or mutant AICE motif probes. CTLA-4 or Bcl11b DNA probes containing base substitution mutations in either the IRF site (IRF mut), the AP-1 site (AP-1 mut), or both (IRF/AP-1 mut) (E); inverted IRF sites (F); or inserted nucleotides between motifs (G) were used in indicated binding reactions (probes in tables S6 and S7). Red and blue arrows mark the positions of the BATF-JunB-DNA and IRF4-BATF-JunB-DNA complexes, respectively. Data are representative of at least two independent experiments.

Enrichment of both motifs in IRF4 targeted regions was highly statistically significant relative to a background frequency estimated by MEME (materials and methods and supplementary text S3). These results suggested that IRF4 is recruited to the composite motifs by interactions with AP-1 family members. Because BATF is an activating protein 1 (AP-1) family transcription factor that is required for T_H17 differentiation (17), we hypothesized that it was the sought-after interaction partner for IRF4 in T_H17 cells. ChIP-seq analysis with BATF in T_H17 cells also showed enrichment for AP-1–IRF composite motifs (4- and 0-bp spacing) that were present in 18% and 16% of the targeted sequences, respectively (fig. S1C). Analysis of coincident peaks (supplementary text S3) revealed that a majority of the IRF4-targeted sequences were co-bound by BATF (Fig. 1B) and delineated both types of composite motifs (Fig. 1C). These data implied that IRF4 is recruited to the distinctive composite elements by a BATF-containing AP-1 complex.

To test whether IRF4 and BATF assemble on the composite motifs, we performed electrophoretic mobility shift assays (EMSAs) by using T_H17 nuclear extracts and DNA probes, representing the alternate configurations (fig. S2, A and B). Both prototype DNA probes gave rise to

co-migrating protein-DNA complexes that contained IRF4 and BATF. These complexes also included JunB, a heterodimeric partner of BATF in T_H17 cells (17). To demonstrate cooperative DNA-dependent assembly, we expressed each protein individually in 293T cells and then used these proteins for EMSAs. No specific protein-DNA complexes were detected with IRF4 alone (Fig. 1D). A complex was observed in reactions containing BATF and JunB. Addition of IRF4 to the BATF-JunB binding reactions resulted in a slower-migrating complex containing all three proteins. Mutational analysis of the IRF and AP-1 sites showed both to be required for the assembly of quaternary complexes with the two types of composite motifs (Fig. 1E). Fine specificity analysis revealed considerable degeneracy in the IRF recognition sequence in the context of IRF4 co-binding with BATF-JunB heterodimers (supplementary text S4). Thus, the AP-1 motif observed by IRF4 ChIP-seq analysis (Fig. 1A) may also be associated with degenerate IRF sites that are not readily revealed by MEME. An AP-1 site was not sufficient to mediate the recruitment of IRF4 to DNA. The latter conclusion was strongly reinforced by use of variant CTLA-4 or Bcl11b probes that differed in the orientation or spacing of the IRF site in relation to the AP-1 site (Fig. 1,

F and G). Thus, binding of a BATF-JunB heterodimer to the AP-1 site functions to recruit IRF4 to DNA in alternate configurations via the latter's recognition of an IRF site in the composite sequence. By analogy with the EICEs characterized in our earlier work (14), we designate the AP-1–IRF composite elements as AICE motifs.

To determine whether IRF4-BATF-JunB complexes bind to presumptive regulatory sequences in genes required by T_H17 cells, we focused on *Il17*, *Il21*, *Il22*, *Il23r*, and *Il12rb1*. Each of these loci was found to contain one or more coincident binding peaks for IRF4 and BATF that were positioned in promoters and/or intronic regions (Fig. 2A, left). ChIP assays not only verified the binding of IRF4 and BATF to these regions but also demonstrated the co-binding of JunB (fig. S3A). Analysis of the DNA sequences underlying these peaks revealed that some had readily discernible AICE motifs whereas others contained AP-1 sites juxtaposed with degenerate IRF sites (Fig. 2A, right, and fig. S3). Nevertheless, all of these sequences promoted the assembly of IRF4-BATF-JunB complexes in T_H17 nuclear extracts (Fig. 2A, right, and fig. S3C). We note that the sequences also varied in their affinity for BATF-JunB complexes (fig. S3, B and D, left). Importantly, IRF4 was seen to reciprocally

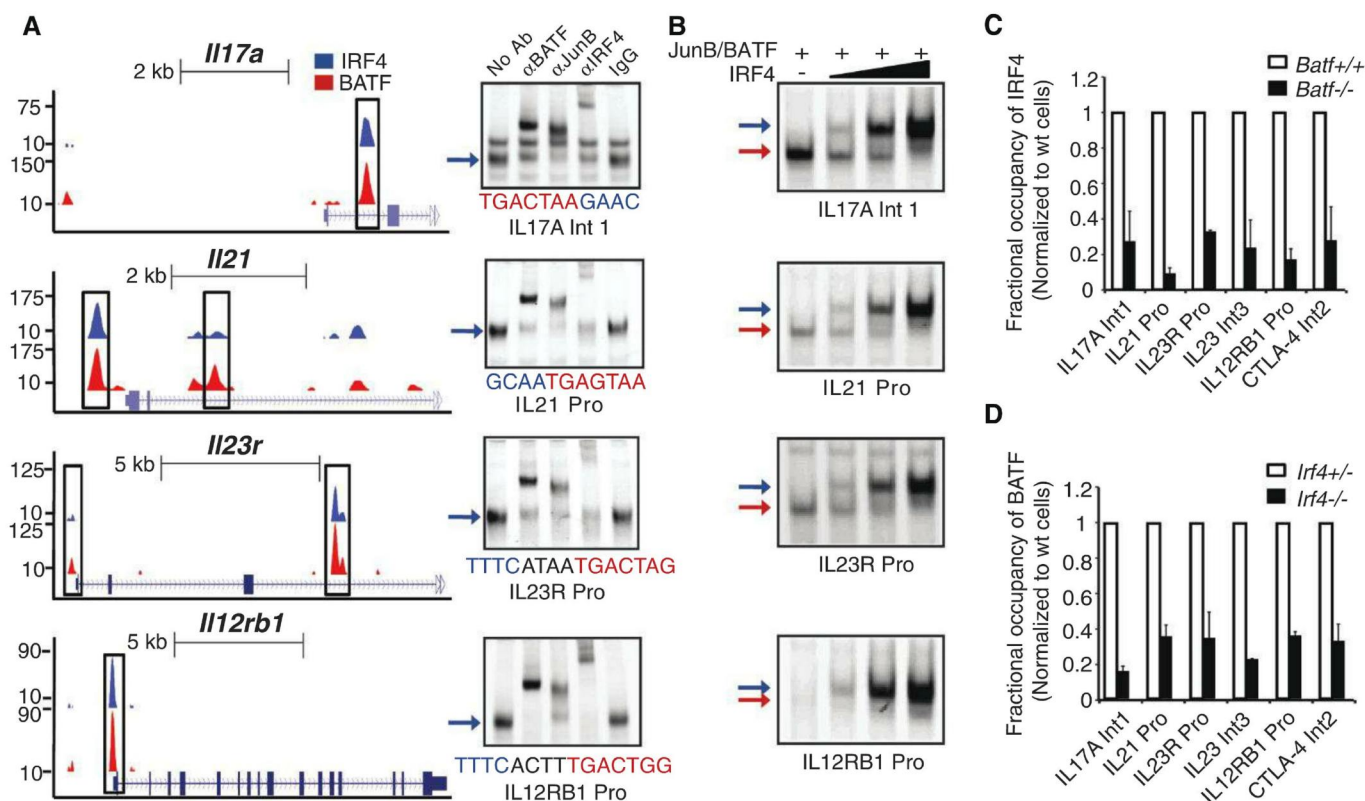


Fig. 2. IRF4-BATF-JunB complexes assemble on presumptive regulatory sequences in key T_H17 genes. (A) (Left) ChIP-seq tracks for IRF4 (blue) or BATF (red) at the *Il17a*, *Il21*, *Il23r*, and *Il12rb1* loci. Coincident peaks containing AICE motifs are highlighted (boxed regions). (Right) EMSAs using nuclear extracts from T_H17 cells and the indicated DNA probes and antibodies. (B) EMSAs using nuclear extracts from 293T cells overexpressing indicated proteins. Reactions were

performed with increasing amounts (fivefold) of IRF4 protein. (C) ChIP analysis of IRF4 binding on indicated sequences in wild-type and *Batf*^{-/-} T cells. (D) ChIP analysis of BATF binding on indicated sequences in *Irf4*^{+/+} and *Irf4*^{-/-} T cells. Purified CD4 cells were polarized for 42 hours under T_H17 differentiating conditions. The average $\pm$ SD of two independent experiments is shown for each target sequence after normalization to control T cells.

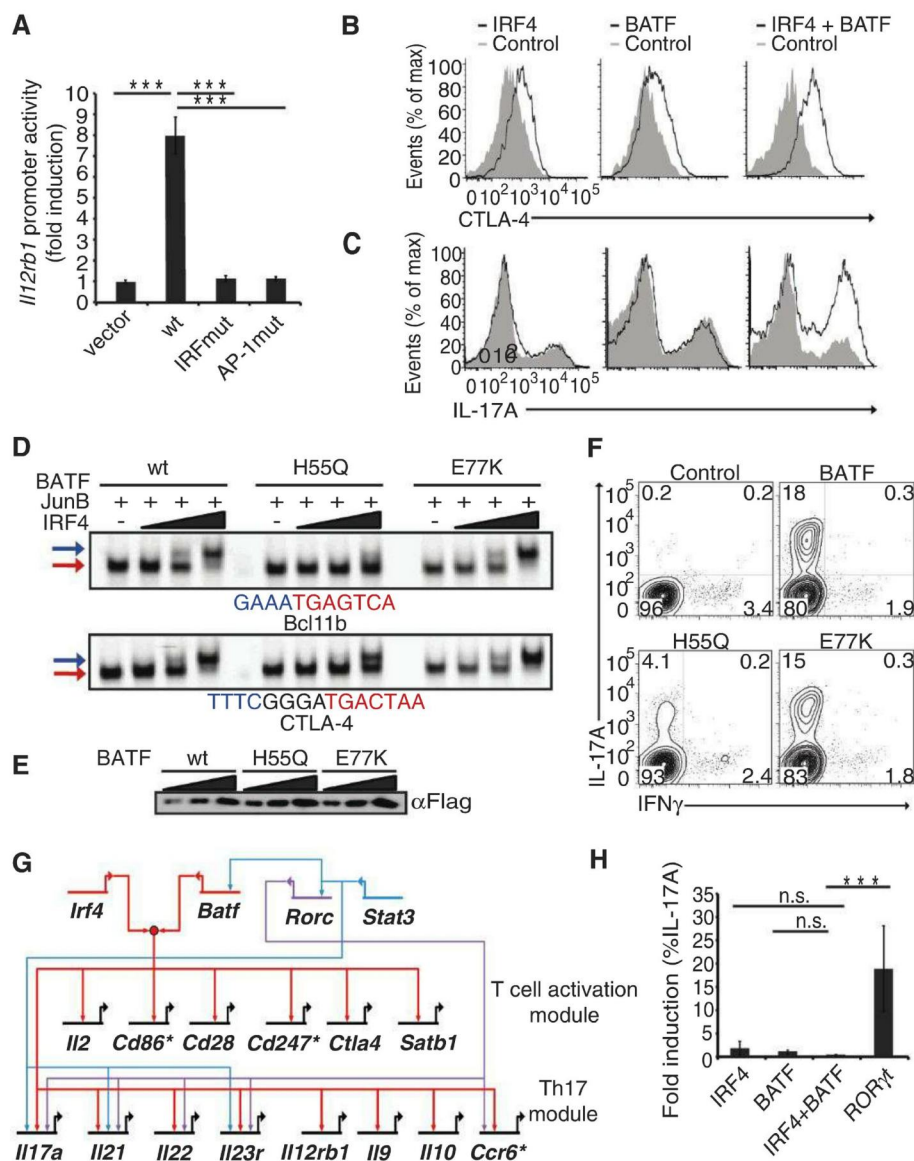
enhance the binding of BATF-JunB (1.5- and 8-fold), more strongly on sequences that had variant lower affinity AP-1 sites (Fig. 2B and fig. S3, E and F). Mutational analysis confirmed the requirement of the AP-1 and IRF sites for DNA assembly (supplementary text S4). Thus, these complexes exhibit the hallmarks of DNA-dependent cooperative binding in spite of structurally divergent composite sites. We next tested whether loss of BATF or IRF4 resulted in impaired binding of the partner transcription factor to target sequences in vivo. IRF4 or BATF binding was reduced on co-targeted sequences in *Batf*^{-/-} or *Ir4*^{-/-} T cells, respectively, cultured under T_H17 polarizing conditions (Fig. 2, C and D). ChIP-seq analyses of *Batf*^{-/-} and *Ir4*^{-/-} T cells revealed that co-targeting of IRF4 and BATF to the *Ctla-4* and *Il12rb1* loci was diminished or lost in the absence of the partner protein (fig. S4 and supplementary text S3). Loss of BATF or IRF4 did not affect the expression

of the partner transcription factor. Therefore, IRF4-BATF-JunB complexes target a key set of T_H17 genes and cooperatively assemble on structurally diverse AICE motifs in vitro as well as in vivo.

Several complementary approaches were used to test functionality of molecular complexes between IRF4 and BATF. Given that the *Il12rb1* promoter contains an AICE motif (Fig. 2), we tested whether its activity in T_H17 cells is dependent on both the AP-1 and the IRF sites. The wild-type promoter displayed significant activity, which was abrogated by mutation of the AP-1 or IRF sites (Fig. 3A). Hence, both the AP-1 and IRF sites are required for the transcription-activating function of an AICE motif. We next performed gain-of-function experiments to determine whether coexpression of IRF4 and BATF promotes activation of target genes containing AICE motifs and the generation of T_H17 cells. CTLA-4 induction was modestly elevated by increasing

IRF4 or BATF levels in nonpolarized CD4⁺ T cells but more substantially by their combined expression (Fig. 3B and fig. S5A). Similarly, IRF4 or BATF overexpression did not appreciably increase the frequency of IL-17A-producing cells, whereas their coexpression resulted in a twofold enhancement (Fig. 3C and fig. S5B). Lastly, by mutational analysis of the BATF leucine zipper region, we were able to identify a residue H55 that participated in the recruitment of IRF4 to AICE motifs (18). Replacement of the histidine with a glutamine residue impaired the recruitment of IRF4 to both types of AICE motifs, with a more severe consequence on its binding to the abutting (Bcl11b) configuration (Fig. 3, D and E). Mutation of a residue Glu⁷⁷ (E77) in the leucine zipper that is distal from the BATF DNA binding domain did not affect complex formation with IRF4. Whereas the wild-type and Glu⁷⁷→Lys⁷⁷ (E77K) BATF proteins robustly restored the generation of T_H17 cells upon their expression in

Fig. 3. IRF4 and BATF complexes activate gene expression and T_H17 differentiation. **(A)** T_H17-polarized cells were transiently transfected with luciferase reporter plasmids containing the wild-type *Il12rb1* promoter or mutant derivatives and a control Renilla luciferase reporter plasmid. Data are from five experiments (average $\pm$ SD) **(B and C)** Naïve CD4 cells activated under nonpolarizing conditions (T_H0) or T_H17-polarizing conditions, respectively, were transduced with the indicated retroviruses. Four days after infection, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hours and analyzed by flow cytometry for intracellular CTLA-4 or IL-17A after gating on transduced cells. **(D)** EMSAs using nuclear extracts from 293T cells overexpressing indicated proteins. Reactions used increasing amounts (fivefold) of IRF4. **(E)** Western blot analysis of wild-type and BATF mutant proteins used in (D). **(F)** *Batf*^{-/-} T cells were activated under T_H17-polarizing conditions and transduced with retroviruses encoding wild-type BATF or indicated mutants. Six days after infection, cells were restimulated under T_H17 conditions and analyzed 5 days later for expression of IL17 and IFN γ by intracellular staining. **(G)** Network diagram of IRF4 and BATF co-targeted and regulated genes. Genes marked with * were differentially regulated in *Batf*^{-/-} T cells based on genome-wide expression analysis but could not be confirmed via quantitative polymerase chain reaction (qPCR). ROR γ t and STAT3 inputs on these genes are indicated in blue. **(H)** T_H0 cells were transduced with indicated retroviruses and analyzed as described in (B). Data are representative of at least two experiments and where indicated include average values $\pm$ SD. n.s., not significant.



Batf^{-/-} T cells, the His⁵⁵→Gln⁵⁵ (H55Q) protein exhibited reduced complementing activity in spite of comparable stability and DNA binding (Fig. 3, D, E, and F). Thus, by mutational analysis of the cis- and trans-acting components of the AICE motif as well as gain- and loss-of-function experiments, these results establish the functional importance of IRF4-BATF complexes in regulation of T_H17 cells.

To identify IRF4-regulated genes among those that are co-targeted by IRF4 and BATF, we performed genome-wide expression analysis of *Ir4*^{-/-} T cells polarized under T_H17 conditions (fig. S6A). There was a profound defect in the expression of *Il17a*, *Il21*, *Il22*, and *Il23r* genes in *Ir4*^{-/-} cells (fig. S6B). Notably, these cells were also defective in expression of *Il12rb1* and *Ccr6* and genes involved in T cell activation, including *Cd86*, *Cd247*, *Cd28*, *Ctla4*, and *Il2*. Of the 362 genes that were positively regulated by IRF4 during T_H17 differentiation, 155 (~42%) contained coincident IRF4 and BATF peaks (table S2). The hypergeometric *P* value for the overall enrichment of IRF4-regulated genes in the IRF4-BATF co-bound data set was <10⁻¹⁶. Ingenuity pathway analysis (IPA) revealed that 65 of the aforementioned 155 genes formed a highly interconnected network (fig. S6C). This network did not include genes encoding the other transcription factors required for T_H17 differentiation, namely, *Rorc*, *Rora*, *Ahr*, and *Stat3* (19–22). However, when these regulatory factors were introduced into the network (fig. S6C), they were seen to make a large number of connections that were statistically significant. Inspection of this network of genes revealed distinct regulatory modules (Fig. 3G). Analysis of this select set of co-targeted and IRF4-regulated genes in

Batf^{-/-} cells showed the majority of them to be dependent on BATF (17) (fig. S6D). Most of these genes contained identifiable AICE motifs in the DNA regions that were co-bound by IRF4 and BATF (fig. S6E). Given that IRF4-BATF complexes appear to co-target and regulate a key set of genes in T_H17 cells that is also controlled by Ror γ t, we tested for their interdependency by gain-of-function experiments in T_H0 cells. As expected, ectopic expression of Ror γ t promoted the generation of T_H17 cells (19) (Fig. 3H). In contrast, individual or coexpression of IRF4 and BATF in T_H0 cells did not enhance T_H17 cell generation. Because Ror γ t is not induced under T_H0 conditions (19), these results suggest that IRF4/BATF complexes function in a combinatorial manner with Ror γ t to induce the expression of T_H17 genes.

Expression profiling of AP-1 family genes in T_H17 cells revealed the inducible expression of c-Jun and FosL2 in addition to BATF and JunB. Therefore, we tested whether alternate AP-1 complexes containing these subunits were capable of cooperatively assembling with IRF4 on AICE motifs. A c-Jun-BATF but not a JunB-FosL2 heterodimer was able to recruit IRF4 to the structurally distinct AICE motifs (Fig. 4, A and B, and fig. S7, A and B). Given a high degree of structural similarity of IRF4 with IRF8, we determined whether the latter factor could also be recruited to AICE motifs by BATF-Jun heterodimers. IRF8 was seen to cooperatively assemble on AICE motifs with BATF-JunB heterodimers (Fig. 4C and fig. S7C). However, this molecular property was not manifested by ubiquitously expressed IRFs, namely, IRF1 or IRF3 (Fig. 4D and fig. S7D). IRF1 and IRF3, unlike IRF4, could bind with high affinity to an ISRE DNA probe (fig. S7E).

Structural modeling and biochemical analysis with the IRF4 DNA binding domain suggest an unusually pliant mode of cooperative DNA binding (fig. S8 and supplementary text S5). Thus, IRF4 and IRF8 are distinguished from other IRFs not only by their immune system-selective expression but also by their unique molecular properties of cooperative assembly with specific members of the Ets and AP-1 superfamilies on EICE and AICE motifs, respectively.

Given the restricted expression and overlapping functions of IRF4 and BATF in T, B, and dendritic cells (5, 6, 17, 18, 23, 24), we used ChIPseq to determine whether IRF4 is able to target AICE motifs in alternate cellular contexts. We analyzed the IRF4 cistromes in activated but nonpolarized T cells (T_H0), IL-4-polarized T_H2 cells, and lipopolysaccharide (LPS)-activated dendritic cells. All three cellular contexts revealed statistically significant enrichment for the AICE motif in targeted sequences (Fig. 4, E to G, and table S3). Because dendritic cells express PU.1 and Spi-B whereas T_H0 and T_H2 cells do not, the EICE motif was observed within the IRF4 cistrome in the former context but was absent in the latter (Fig. 4G and table S3). As is the case for dendritic cells, the IRF4 cistrome in B cells was also seen to contain EICE as well as AICE motif targeted regions (supplementary text S6). A pairwise statistical analysis of the use of AICE and EICE motifs in the various immune cell contexts confirmed their distinctive distributions in IRF4-targeted sequences (table S4). These results suggest that, although IRF4 functions with BATF-containing AP-1 heterodimers on the AICE motif in both adaptive and innate immune cell contexts, T_H17 and T_H2 cells rely exclusively on such complexes.

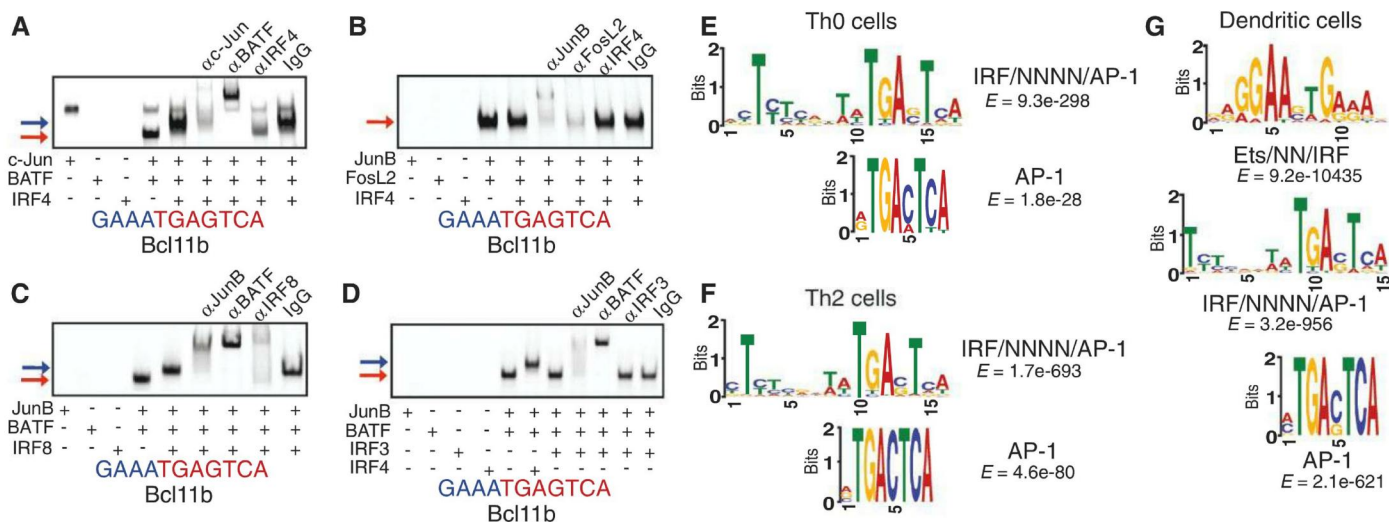


Fig. 4. Specificity of AP-1-IRF complexes that cooperatively assemble on AICE motifs. (A to D) EMSAs using the Bcl11b probe and nuclear extracts from 293T cells overexpressing the indicated proteins: (A) c-Jun, BATF, IRF4; (B) FosL2, JunB, IRF4; (C) JunB, BATF, IRF8; or (D) JunB, BATF, IRF3, and IRF4. Complexes were supershifted with indicated antibodies. Red and blue arrows mark the ternary and quaternary protein-DNA complexes, respectively, as in Fig. 1. Data

are representative of at least two independent experiments. (E to G) ChIPseq analysis of in vitro differentiated T_H0 and T_H2 cells (42 hours) and LPS-activated dendritic cells (6 hours) with antibodies directed against IRF4. Highly represented motifs and their respective *E* values within the IRF4 cistrome in T_H0 (241 peaks), in T_H2 (725 peaks), and in dendritic cells (10,364 peaks) are displayed.

We propose that the AICE motif is an immune-specific genomic regulatory element that is widely used in both innate and adaptive immune cells. Its functioning is predicted to be largely confined to cells of the immune system by virtue of the restricted expression of IRF4 and IRF8 as well as BATF or its paralogs (18). The involvement of the AICE motif in distinct innate or adaptive immune cell-specific programs of gene expression, as exemplified in T_H17 cells, is likely to reflect the combinatorial functioning of AP-1–IRF4 or AP-1–IRF8 complexes with differentiation state-specific regulators such as Ror γ t in T_H17 cells or Gata3 in T_H2 cells. In addition to its T_H effector state-specific functions, the AICE motif also regulates key components within the T cell activation program. Given that AP-1 family members and IRF4 and IRF8 are signaling-induced transcription factors (2, 25), the AICE motif appears to have evolved to sense and integrate diverse signaling inputs in immune cells. It is capable of integrating such inputs from antigen receptors and their co-receptors as well as cytokine receptors and TLRs. Because AP-1 family members can cooperatively assemble on composite elements with nuclear factor of activated T cells (NFAT) family proteins (26) and juxtaposition of NFAT and IRF sites has been reported on the IL-4 and IL-10 genes (8, 27), it will be important to determine whether the AP-1–IRF4 or AP-1–IRF8 complexes described herein can cooperatively assemble with NFAT proteins, thereby enabling the integration of Ca signaling in the regulatory module. We propose that the acquisition of the

AICE motif by simple variation of AP-1 sites in biologically important immune response genes may have provided a selective force for the evolution of the structurally divergent and specialized members of the IRF family, namely, IRF4 and IRF8.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1228309/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S8
Tables S1 to S8
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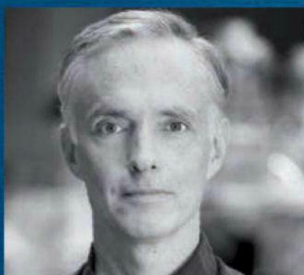
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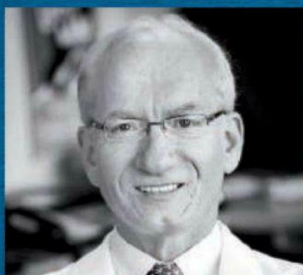
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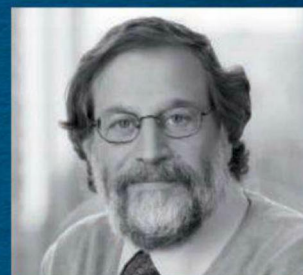
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Professor and Head of the
Leonard Wagner Laboratory
of Molecular Genetics and
Immunology at
the Rockefeller University.

SANOFI - INSTITUT PASTEUR AWARDS FOR YOUNG BIOMEDICAL SCIENTISTS

Sanofi and the Institut Pasteur
decided to honour two exceptional
young scientists selected for their
excellent biomedical research.



Dr STÉPHANIE BLANDIN

Researcher, Inserm
Molecular and Cellular
Biology Institute,
Strasbourg.



Dr PHILIPPE BOUSSO

Research Director, Inserm
Head of Dynamics of
Immune Responses,
Institut Pasteur, Paris.

Photography credits: Andrius Jackimaitis (top) - Franck Parisot (bottom)

MICHIGAN STATE UNIVERSITY

Microbial Ecology/Environmental Microbiology Department of Microbiology and Molecular Genetics

The Department of Microbiology and Molecular Genetics at Michigan State University seeks candidates for an academic-year position at the Assistant or Associate Professor level in microbial ecology or environmental microbiology, broadly defined. We are particularly interested in applicants with demonstrated expertise and interest in the ecology, physiology and interactions of microbes in aquatic systems, and their dynamics and evolution. Candidates working with non-aquatic microbial systems will also be considered. Many opportunities exist for collaboration within the Center for Water Sciences; the Center for Microbial Ecology; the BEACON Center (Evolution in Action); and the Enterics Research Investigational Network. The successful candidate will have a PhD in microbiology or a related field, as well as postdoctoral experience. A strong record of research accomplishment is expected with potential to have an independent, externally funded research program with national visibility. Academic rank will be commensurate with experience. The offer will include a competitive startup package and laboratory facility.

Applications should be submitted electronically through the MSU Applicant Page (MAP) website at <https://jobs.msu.edu> (position #6843). Applicants must upload a single pdf file containing a letter of interest; a history of funding; a statement of future research; curriculum vitae; and the names of at least three potential references. Review of applications will begin immediately. The position will remain open until filled.

Additional information can be obtained via email to the Search Committee Executive Assistant at mmgchair@msu.edu or at our website <http://www.mmg.msu.edu>.

MSU is an Affirmative Action, Equal Opportunity Employer. MSU is committed to achieving excellence through cultural diversity. The University actively encourages applications and/or nominations of women, persons of color, veterans and persons with disabilities.

MICHIGAN STATE UNIVERSITY

Microbial Ecology of Infectious Disease Department of Microbiology and Molecular Genetics

The Department of Microbiology and Molecular Genetics at Michigan State University seeks candidates for an academic-year position at the Assistant or Associate Professor level position in microbial ecology with an emphasis on the microbiome and/or related infectious diseases. We are particularly interested in applicants with an emphasis on the microbiome, infectious diseases, beneficial organisms, and microbial systems related to water. Candidates working in other systems will be considered. Many opportunities exist for collaboration with faculty with research interests in the NSF BEACON Center (Evolution in Action); the Center for Microbial Ecology; the NIH Enterics Research Investigational Network; and the Center for Water Sciences. A strong record of research accomplishment and development of an independent, externally funded research program with national visibility is expected. Academic rank will be commensurate with experience. The offer will include a competitive startup package and laboratory facility.

Applications should be submitted electronically through the Human Resources (MAP) website at <https://jobs.msu.edu> (position #6857). Review of applications will begin immediately. The position will remain open until filled. Applicants must submit the following application items on the website above in one pdf file containing a letter of interest; a history of funding; a statement of future research; curriculum vitae; names of three potential references. Review of applications will begin immediately. The position will remain open until filled.

Additional information could be obtained via email to the Search Committee Executive Assistant at mmgchair@msu.edu. <http://www.mmg.msu.edu>

MSU is an Affirmative Action, Equal Opportunity Employer. MSU is committed to achieving excellence through cultural diversity. The University actively encourages applications and/or nominations of women, persons of color, veterans and persons with disabilities.



Assistant/Associate Professor in Cancer Biology

Indiana University School of Medicine – Medical Sciences in Bloomington (<http://bloomington.medicine.iu.edu>) invites applications for a 12-month tenure-track position in cancer biology. While all areas of research will

be considered, we are especially interested in candidates focusing on epigenetics or cancer genomics. The successful applicant will join faculty from Medical Sciences and the College of Arts and Sciences (Biology, Biochemistry, Chemistry), and will have the opportunity to develop collaborations with faculty in the Melvin and Bren Simon Cancer Center, the School of Informatics and Computing in Bloomington, and the Center for Computational Biology and Bioinformatics in Indianapolis. The candidate will have access to state of the art facilities for genomics, bioinformatics, biological imaging, protein analysis and structural biology. Successful applicants must have a PhD, MD or MD/PhD, demonstrate a strong potential to establish an externally funded research program, and contribute to the departmental teaching mission at the undergraduate, graduate, or medical level.

Full review of applications will commence **December 1, 2012**, and continue until the position is filled. Applicants should send a single PDF file containing curriculum vitae; statement of research interests and directions; statement of teaching philosophy; and summary of current and anticipated research support to cancerbi@indiana.edu. Three letters of reference should be sent under separate cover to cancerbi@indiana.edu.

Indiana University is an Equal Opportunity/Affirmative Action Employer. Women and minorities are encouraged to apply.

THE UNIVERSITY OF TEXAS MD Anderson Cancer Center

Making Cancer History®

The Department of Experimental Therapeutics seeks an exceptionally qualified basic/translational science applicant for a tenure-track appointment as an assistant/associate professor.

The successful candidate will possess an outstanding ability to conduct independent, high-quality translational cancer research with a proven track record of publications and peer-reviewed funding. The position also will be responsible for building a world-class laboratory research program developing tumor cell-targeting biomolecules (such as growth factors, antibodies, targeting scaffolds or oligonucleotides) that can serve as carriers of therapeutic and imaging payloads. A generous start-up package is available. This program will be an essential component of the new Center for Targeted Therapy at MD Anderson Cancer Center.

Applicants must hold a Ph.D. and/or M.D. in a basic science-related field. The position requires a highly motivated and independent candidate who will establish collaborative interactions with clinicians and translational researchers developing new cancer therapies. Candidates also should be prepared to carry out education and mentoring activities with graduate students and postdoctoral fellows.

The University of Texas MD Anderson Cancer Center offers generous start-up funds; quality laboratory space; and the opportunity to collaborate with leading scientists in molecular target identification, pharmacology, biological therapy, drug development and clinical testing throughout the department and institution.

Please forward curriculum vitae; contact information for references; and a brief statement, including accomplishments, current and proposed research objectives and plans to emerge as a leader in chosen area, to: ETDept@mdanderson.org

MD Anderson Cancer Center is an equal opportunity employer and does not discriminate on the basis of race, color, national origin, gender, sexual orientation, age, religion, disability or veteran status except where such distinction is required by law. All positions at The University of Texas MD Anderson Cancer Center are security sensitive and subject to examination of criminal history record information. Smoke-free and drug-free facility.

Assistant/Associate Professor Targeted Biomolecules

Michael G. Rosenblum, Ph.D.
Head of the Search Committee
Department of Experimental
Therapeutics – Unit 1950
The University of Texas
MD Anderson Cancer Center
P.O. Box 301429
Houston, Texas 77230



Chair, Department of Biochemistry

Northwestern University Feinberg School of Medicine invites applications and nominations for the position of Chair, Department of Biochemistry.

Assuming leadership at the Feinberg School of Medicine, the Chair will join Feinberg at a crucial point in time with the formation of our national brand called Northwestern Medicine. The Chair is not just the department leader but also an important figure across the academic medical center and will build upon existing institutional strengths to lead the formation of new Department of Biochemistry with space and opportunity to recruit substantial numbers of new faculty.

Principal investigators appointed through the Feinberg School of Medicine, ranked 18th in *U.S. News & World Report*, are supported by \$371 million of annual research funding. The Chair will report directly to the Dean of the medical school. Successful candidates will possess a PhD or equivalent degree, be eligible for appointment as a full-time Professor, and have a record of scholarly accomplishments and national recognition in biochemistry.

Please email nominations and CVs of appropriate candidates to:

Patricia Spear, PhD
p-spear@northwestern.edu, chair of the search committee

and copy Ila Allen
ila@northwestern.edu, recruitment coordinator, Feinberg School of Medicine.

Applications will be taken until the position is filled. Northwestern University is an Affirmative Action, Equal Opportunity Employer. Women and minorities are encouraged to apply. Hiring is contingent upon eligibility to work in the United States.



Faculty Positions Department of Neuroscience

Scripps Florida is seeking outstanding applicants for tenured faculty positions at all levels in the Department of Neuroscience at its newly opened, state-of-the-art campus in Jupiter, Florida. The Jupiter campus of the Scripps Research Institute offers a rapidly growing, dynamic environment and is committed to provide a multi-disciplinary, collaborative atmosphere for fostering cutting edge neuroscience research. We invite applications from ambitious and interactive investigators applying integrative molecular, genetic, biochemical, biophysical, cellular (iPSC), anatomical, and behavioral approaches to elucidate the mechanisms underlying nervous system function. In addition to basic neuroscience, we have a keen interest in human neuropsychiatric disorders, animal models for these disorders, and CNS drug discovery. The state-of-the-art facilities at Scripps Florida house expertise in proteomics, crystallography, pharmacokinetics, medicinal chemistry, rodent behavior, and ultra high-throughput small molecule screening for drug discovery.

Scripps Florida offers exceptional startup packages and an outstanding intellectual environment for fostering top-tier basic and translational research. Further information about our expanding Department of Neuroscience can be found at <http://www.scripps.edu/florida/neuro/>. Interested candidates should submit their *Curriculum Vitae* and contact information for at least three professional references as a single PDF file, to:

Dr. Ronald L. Davis, Chairman, Department of Neuroscience
 c/o Trina Kemp
 (tmiles@scripps.edu)

The Scripps Research Institute, Scripps Florida
 130 Scripps Way
 Jupiter, Florida 33458

TSRI embraces diversity and recognizes it as being a key to our success.
 EOE/M/F/V/D.



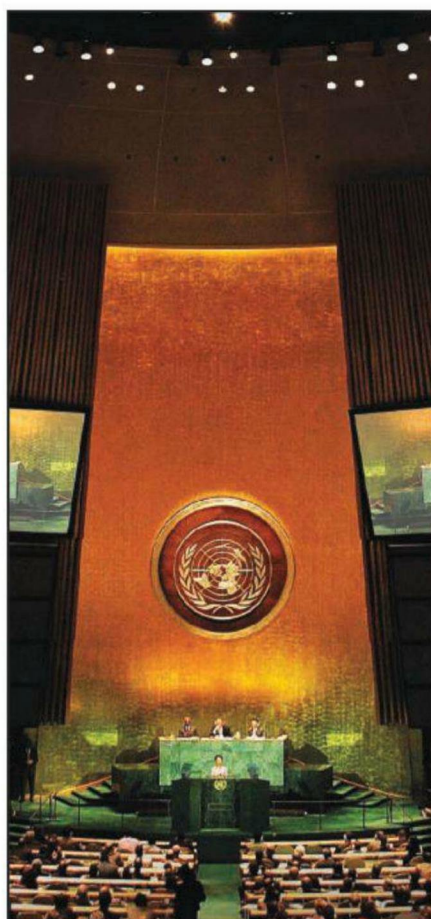
FACULTY POSITIONS MEDICAL SCIENCES

Washington State University Division of Health Sciences, located on the Riverpoint campus in Spokane, invites applications for two tenure-track faculty positions at the rank of Assistant or Associate Professor and one at the rank of Professor in its new Medical Sciences section. Applicants must have an earned doctorate degree in the basic, clinical, or translational medical sciences. Successful candidates will be expected to maintain an active, extramurally funded research program, to mentor graduate students and fellows, and to teach in the professional and/or graduate curricula. The successful candidate for the Professor position will additionally be expected to take a leadership role in rapidly expanding medical research in Spokane, including recruitment of faculty and programmatic development.

Areas of research interest are open, but preference will be given to candidates completing research in the areas of planned growth in our program including molecular, cellular, physiological and systems biology approaches to: neurosciences/behavioral neurosciences (sleep, addictions, pain, anesthesia), senescence and immortality (stem cells, cancer, aging, regenerative medicine), microbiology (antimicrobial resistance, virology), and metabolic diseases (obesity, diabetes, renal and cardiovascular disease). Washington State University is substantially building its research and graduate education capacity in the medical sciences. The Medical Sciences section also participates in preclinical medical education in the WWAMI program, which is a collaborative medical education program with the University of Washington School of Medicine.

Screening of applications will begin immediately and will continue until a suitable candidate is identified. To apply visit: www.wsujobs.com. Applications must include a current curriculum vitae and letter of application describing professional goals, research, and teaching experience. Before interviews commence four letters of reference will be required. **Contact Kim Noe, Administrative Manager**, at knoe@wsu.edu or 509-358-7515 for questions, assistance with the application process or confidential expressions of interest.

Women and minorities are particularly encouraged to apply. Washington State University Is An Equal Opportunity/affirmative Action Educator And Employer.



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The California NanoSystems Institute (CNSI) at the University of California-Santa Barbara is pleased to announce the 2013 competition for the **ELINGS PRIZE FELLOWSHIPS in EXPERIMENTAL SCIENCE**

The Elings Prize postdoctoral fellowships provide a salary of \$60,000/year for two years, renewable for a third, with benefits and research funds. Eligible research can be in any area of the physical sciences, biology, or engineering. Successful applicants will work with, and receive partial support from, experimentalists on the CNSI faculty; applications must be coordinated with the relevant faculty supervisor(s). For more information, visit http://www.cnsi.ucsb.edu/admin/funding/elings_fellowships/.

Applicants holding a PhD in science or engineering should submit a cover letter, curriculum vitae, a one-page research proposal, and arrange for 3 supporting letters, all submitted via the website <https://fellow.cnsi.ucsb.edu>. The deadline for applications is **January 31, 2013**.

The University of California is an Equal Opportunity/Affirmative Action Employer.



THE UNIVERSITY OF CHICAGO

Academic Career Opportunities Faculty Position Req # 01475

The Department of Biochemistry and Molecular Biology at The University of Chicago invites applications for positions on its faculty at any rank. We are interested in scientists with outstanding accomplishments in and potential for fundamental research in biochemistry and/or molecular biophysics, broadly defined, and a zest for teaching. The interests of these individuals should synergize with the research in this department and other relevant biomedical programs. Generous start-up support will be provided to prime a robust research program.

Applications must include a curriculum vitae that details all publications and accomplishments, as well as plans for future research, and a teaching statement. Applications must be submitted on-line by **December 14, 2012** to academiccareers.uchicago.edu/applicants/Central?quickFind=52517. Those applicants for appointment as Assistant Professor should arrange for three letters of reference to be sent to the **Search Committee, Department of Biochemistry and Molecular Biology, 929 E. 57th Street, Room W225, Chicago IL 60637** or to BMB@bsd.uchicago.edu.

The University of Chicago is an Affirmative Action / Equal Opportunity Employer.



The University of Texas at Dallas
Department of Molecular
and Cell Biology

FACULTY POSITIONS

The Department of Molecular and Cell Biology of the University of Texas at Dallas (<http://www.utdallas.edu/biology/>) invites applications for Faculty positions at any rank. Preference will be for investigators working in research areas that complement existing Departmental strengths in biochemistry, cancer biology, cell biology, computational and systems biology, microbiology, and neurobiology. Applicants should show evidence of a vigorous and independent research program, and those at the Associate or Full Professor levels should also have a record of obtaining significant external funding. Applicants should have enthusiasm for teaching at both graduate and undergraduate levels.

Review of applications will begin immediately and will continue until the positions are filled. Indication of gender and ethnicity for affirmative action statistical purposes is requested as part of the application.

Applicants should submit a curriculum vitae, short descriptions of research plans and teaching interests, and three letters of recommendation via the ONLINE APPLICATION FORM available at <http://go.utdallas.edu/pnf121029>

The University of Texas at Dallas is an Equal Opportunity/Affirmative Action Employer. All qualified applicants will receive consideration for employment without regard to race, color, religion, sex, national origin, disability, age, citizenship status, Vietnam era or special disabled veteran's status, or sexual orientation.

MacArthur Foundation

Senior Program Officer The MacArthur Fellows Program

The MacArthur Foundation is seeking a Senior Program Officer who will be assigned nominations mainly (but not exclusively) in natural sciences, technology, engineering, mathematics, and medicine. In addition, the Senior Program Officer will provide informal guidance to other program officers who are working on nominations in related areas. The Senior Program Officer will also contribute to the development of lists of potential nominators, and actively follow recent advances in fields across the spectrum of scientific research.

For a complete job description and to apply, candidates should visit www.macfound.org/jobs. (Electronic submissions are required.) No Phone Inquiries.

EOE

UC DAVIS

DIRECTOR
UC DAVIS CENTER FOR
COMPARATIVE MEDICINE
School of Medicine and
School of Veterinary Medicine
University of California, Davis
Davis, California 95616

Applications and nominations are invited for the position of Director, UC Davis Center for Comparative Medicine (<http://cem.ucdavis.edu>). The Director will hold a joint administrative and professorial appointment in appropriate departments of the School of Medicine and School of Veterinary Medicine.

The Center is a cooperative venture between the Schools of Medicine and Veterinary Medicine that is housed in a fully equipped research building with an active funding portfolio of over \$97,000,000. Research programs of the 10 core faculty within the Center focus on the pathogenesis of human disease using experimental animal models, with an emphasis on infectious disease. Current areas of investigation include: (1) viral and bacterial diseases [AIDS, Lyme disease, salmonellosis, cytomegalovirus disease, influenza, *Helicobacter pylori*, tuberculosis]; (2) vaccine and anti-viral drug development; and (3) cancer biology. As part of its animal modeling mission, the Center oversees the UC Davis Mouse Biology Program (<http://mouse.ucdavis.edu>). The Center is also strongly integrated with programs at the adjacent California National Primate Research Center.

The Director is responsible for developing and continuing the vision of this unique partnership between the Schools of Medicine and Veterinary Medicine focused on interdisciplinary approaches to animal models of human disease. Responsible for management of the Center; specific responsibilities include: (1) administration of the overall program and facilities of the Center, (2), integration of the Center into the teaching, research and service programs of the two Schools, (3) design and implementation of a development program, (4) participation in instructional programs of the two Schools, and (5) other activities related to integrating the Center into the larger UC Davis research mission. In addition, the successful candidate must maintain an active, extramurally funded animal model-based disease research program.

The University of California is one of the largest and most renowned centers of higher education in the world. The faculty of the University are internationally known for their distinguished academic achievements. UC Davis is ranked 10th nationally among public universities in research funding. The campus lies adjacent to the city of Davis, 14 miles west of Sacramento, 72 miles northeast of San Francisco and 110 miles southwest of Lake Tahoe and the Sierras. The city of Davis has a population of 63,000 and is situated in a beautiful pastoral setting. The University's student population is approximately 32,000.

Candidates must have (1) MD, DVM, PhD or combination of these degrees; (2) a broad understanding of modern biomedical science; (3) demonstrated administrative/leadership skills; (4) an active and distinguished research record in the use of animal models to elucidate the mechanisms, prevention and/or treatment of human disease; (5) demonstrated excellence in teaching; (6) a nationally recognized reputation for career achievement and leadership; and (7) experience in the acquisition of extramural funding, including active development of programmatic grants, and a commitment to work in securing additional funding for the programs of the Center. The Director is expected to qualify for and be appointed as a tenured Professor in an appropriate department in the School of Medicine and/or the School of Veterinary Medicine.

Applicants should submit a letter of interest, a curriculum vitae and the names and addresses of three professional references to **Christine Herkenrath, Assistant Director, Center for Comparative Medicine, University of California, Davis, CA 95616-8734; (email: cmherkenrath@ucdavis.edu; telephone: 530-752-1245; fax: 530-752-7914)**. Nominations should be sent to the same address. For full consideration please submit by **February 1, 2013**, however, applications will be accepted until an individual has been selected.

UC RIVERSIDE

UNIVERSITY OF CALIFORNIA

Department of Entomology
College of Agricultural and Natural Sciences

Assistant Professor and Assistant Entomologist in the area of Molecular Insect Physiology

The Department of Entomology invites applications for an Assistant Professor and Assistant Entomologist in the area of Molecular Insect Physiology at the University of California, Riverside. Position available July 1, 2013, tenure-track position, 9-month appointment, 25% IR/75% OR. Appointment level and salary commensurate with experience. Ph.D. in Entomology, Molecular Biology, Physiology or a related discipline is required; post-doctoral experience is preferred. We are interested in applicants that use integrative approaches including modern molecular, genetic, genomic and biochemical tools in their approaches to understanding of key physiological functions of insects or related arthropods. The development of the application of such knowledge toward the development of new strategies to control insect pests of agricultural crops and vectors of human and animal diseases is encouraged. Teaching responsibilities include supervision of graduate students, participation in undergraduate instruction including instruction in insect physiology, and shared teaching of graduate level courses in insect physiology and molecular biology. The appointee will be encouraged to develop a specialized course in appropriate areas of expertise. Interactions with the other research groups in interdepartmental programs are encouraged.

Send a curriculum vitae, statements of research interests, teaching interests and philosophy, up to three select reprints of publications, manuscripts in press, and arrange for four confidential letters of recommendation to be sent to: **Dr. Alexander Raikhel, Molecular Insect Physiology Search Committee Chair, Department of Entomology, University of California, Riverside, CA 92521; E-mail: alexander.raikhel@ucr.edu; phone: 951-827-2129**. Review of applications will begin **January 15, 2013**, but the position will remain open until filled. Information about the Entomology Department and an expanded description can be found on the website: <http://www.entomology.ucr.edu>.

Assistant Professor and Assistant Entomologist in the area of Arthropod Symbiont Interactions

Department of Entomology invites applications for an Assistant Professor and Assistant Entomologist in the area of Arthropod Symbiont Interactions at the University of California, Riverside. Position available July 1, 2013, tenure-track position, 9-month appointment, 25% IR/75% OR. Appointment level and salary commensurate with experience. Ph.D. in Entomology, Biology, Microbiology, Ecology or a related discipline is required; post-doctoral experience is preferred. The focus of this position will be on studying interactions between symbionts and their arthropod hosts. Emphasis will be placed on the use of modern techniques to elucidate how these interactions shape the biology, ecology, evolution, and behavior of arthropods and their symbionts. Applied and basic research consistent with the mission of the Agricultural Experiment Station directed toward managing arthropod pests and/or vectors of diseases is expected. Teaching responsibilities include supervision of graduate students, participation in undergraduate instruction (e.g. entomology, microbiology, ecology, or evolution), as well as a graduate course taught in an area of interest. Interactions with the other research groups in interdepartmental programs are encouraged.

Send a curriculum vitae, statements of research interests, teaching interests and philosophy, up to three select reprints of publications, manuscripts in press, and arrange for four confidential letters of recommendation to be sent to: **Dr. Richard Stouthamer, Arthropod Symbiont Interactions Search Committee Chair, Department of Entomology, University of California, Riverside, CA 92521; E-mail: richard.stouthamer@ucr.edu; phone: 951-827-2422; fax: 951-827-3086**. Review of applications will begin **March 15, 2013**, but this position will remain open until filled. Information about the Entomology Department and an expanded description can be found on the website: <http://www.entomology.ucr.edu>.

The University of California is an Affirmative Action/Equal Opportunity Employer committed to excellence through diversity, and strongly encourages applications from all qualified applicants, including women and minorities.

http://affirmativeaction.ucr.edu/forms/eeo_survey.html

Women in Science Booklet

Science and the L'Oréal Foundation present



Read inspiring profiles of women
making a difference in biology.

Free download at
ScienceCareers.org/LOréalWIS

ASSISTANT PROFESSORS

Department of Biological Sciences

The University of Alabama is among the top academic research institutions in the southeastern United States, and the Department of Biological Sciences is committed to maintaining this tradition of excellence. We currently seek applicants for tenure-track faculty positions at the rank of **Assistant Professor** in (1) **Computational Biology**, and (2) **Microbial Biology**.

Computational Biology

All areas of computational biology and bioinformatics will be considered. Applications from candidates with a demonstrated record of developing and/or applying computational approaches to study biological questions in areas including comparative genomics and transcriptomics, evolutionary genomics, phylogenomics, genetics/population genetics, cell and molecular biology, and systems biology and a demonstrated interest in collaborative research are especially encouraged to apply. Candidates must have a Ph.D. in the biological sciences or related field, and postdoctoral experience. The successful applicant will be expected to develop an active, externally funded research program and participate in the undergraduate core curriculum in addition to teaching upper-level undergraduate and graduate courses. Applicants may contact the chair of the computational biology search committee, Dr. Julie Olson at olson@bama.ua.edu or 205-348-2633, if additional information is required.

Microbial Biology

All areas of microbial biology will be considered. Applicants conducting research in the areas of microbial systems biology, stress response mechanisms, host-microbe interactions, and microbial genetics using genomics, proteomics, and/or transcriptomics approaches are particularly encouraged to apply. Candidates must have a Ph.D. degree in the Biological Sciences or related field of study, postdoctoral experience, and a strong publication record. The successful applicant will be expected to develop an active, externally funded research program, interact with and enhance existing research groups in the department, and have an interest in developing quality instruction at the undergraduate and graduate levels, with course responsibilities within areas of expertise and departmental needs, including courses in the Microbiology core curriculum, such as General Microbiology and Microbial Genetics. The ideal candidate will also demonstrate the potential to develop a collaborative, multidisciplinary research program. Applicants may contact the chair of the Microbial Biology Search Committee, Dr. Stevan Marcus, at smarcus@as.ua.edu or 205-348-8094, if additional information is required.

To apply, go to <https://facultyjobs.ua.edu>, complete the online application (Job #0807478) or (Job #0807485), and upload (1) an application letter with a list of at least four references (including contact information) under "Other Document 1"; (2) CV; (3) statement of research interests and goals; and (4) statement of teaching interests and philosophy. Consideration of applications will begin December 15, 2012, and continue until the positions are filled. Prior to the hiring, the final candidate(s) may be required to successfully pass a pre-employment background investigation.

Additional information on the Department of Biological Sciences and the available positions can be found on our website at <http://bsc.ua.edu>.

The University of Alabama is an Equal Opportunity/Equal Access Employer and actively seeks diversity among its employees. Women and minorities are encouraged to apply.

touching lives
THE UNIVERSITY OF ALABAMA

Chair, Department of Biomedical Engineering

UNIVERSITY OF MICHIGAN, ANN ARBOR

The Department of Biomedical Engineering at the University of Michigan invites nominations and applications for the position of Department Chair.

The Department, currently ranked 7th by *US News and World Report*, is now being jointly administered by the College of Engineering and Medical School. It currently has 19 tenured and tenure-track faculty members, with a broad portfolio of externally funded research programs covering the full spectrum of biomedical engineering. The Department seeks to significantly grow its faculty during the next three to five years.

The successful candidate will be an outstanding scholar with an earned doctorate in a field related to Biomedical Engineering and an exemplary record of achievement in research, teaching and service at a level commensurate with appointment as a tenured full professor. He or she must also possess visionary leadership abilities, a broad appreciation for the diverse perspectives within biomedical engineering, and a strong interest in promoting sponsored research programs and mentoring faculty. The successful candidate should also be pre-eminently qualified to lead a substantial development effort, catalyze and steward research and educational initiatives across departmental and college boundaries, particularly between the College of Engineering and the Medical School, as well as interact with government, industry, professional societies and alumni, and lead and support a diverse group of faculty, staff and students.

Applicants should electronically submit a detailed curriculum vitae and a two-page synopsis of his or her views on the current challenges and opportunities facing biomedical engineering education and research to: **Professor Shuichi Takayama, Chair, BME Chair Search Advisory Committee**, at bmeseach2012@umich.edu. **Inquiries and nominations may also be directed to Professor Takayama at takayama@umich.edu, or 734-615-5539.**

The University of Michigan is a premier public university with top-rated Engineering, Medical, Law and Business programs, and is responsive to the needs of dual career families. The College of Engineering and the University of Michigan Medical School are dedicated to the goal of building a culturally and intellectually diverse environment. The College of Engineering and the Medical School have substantial research collaboration in addition to the joint Biomedical Engineering Department.

For further information about the College of Engineering, go to: <http://engin.umich.edu/>. For more information about the University of Michigan Medical School, go to: <http://www.med.umich.edu/medschool/>.



MICHIGAN ENGINEERING

UNIVERSITY of MICHIGAN ■ COLLEGE of ENGINEERING



AAAS is here – helping scientists achieve career success.

Every month, over 400,000 students and scientists visit ScienceCareers.org in search of the information, advice, and opportunities they need to take the next step in their careers.

A complete career resource, free to the public, *Science Careers* offers a suite of tools and services developed specifically for scientists. With hundreds of career development articles, webinars and downloadable booklets filled with practical advice, a community forum providing answers to career questions, and thousands of job listings in academia, government, and industry, *Science Careers* has helped countless individuals prepare themselves for successful careers.

As a AAAS member, your dues help AAAS make this service freely available to the scientific community. If you're not a member, join us. Together we can make a difference.

To learn more, visit aaas.org/plusyou/sciencecareers



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The city of Guangzhou welcomes you, a place where you can innovate and establish new business



To find out more info about overseas recruitment and new business opportunities in Guangzhou, please visit following web sites: <http://www.gzhwrc.com>
<http://www.gzscse.gov.cn>

Oversea Professional Service Hotlines:
+8620-83568037 +8620-83568051
Email: wgzjb@gzscse.gov.cn



— Beautiful city of Guangzhou

Guangzhou, also known as "City of Goat", "City of Flowers" is located in the southern part of China. Due to its close proximity to Hong Kong and Macau, it is also known as the window to the China's economic reforms. The city enjoys great weather throughout the year, and spring seems never end. The beautiful Baiyun Mountain and Zhujiang River together help to define city's unique landscape.

— International Hub for professionals

For the past many years, Guangzhou has experienced great economic growth; in 2011 the GDP for the city was CNY 1238

billion. It has also established a CNY 200million fund to support the overseas professional and experts in the region. The fund is used to assist them in areas such as career advancement, local filling and registration services, housing allowance, medical expense, school for children, employment for spouse etc. CNY 5 million would be available for expatriates to start new business; 300 thousand to 1 million would be available for them to settle down in Guangzhou.

— High growth potential

As the capital of the Guangdong province, Guangzhou is one of the five national central cities in China. It is the economic, scientific, academic and

cultural center of south China. Guangzhou is home of many largest companies in China. 7 companies listed in China's top 500 corporations in Guangzhou are also recruiting overseas now. Many other corporations are also eagerly looking for overseas professionals; today over 300 companies has posted more than 1000 positions on the Guangzhou overseas recruitment website -<http://www.gzhwrc.com>

— Diversified Entrepreneurship Platform

2 national level economic development zones: the Guangzhou Development Zone and Zeng Cheng Development Zone.
1 New Business Development Zone: the Nansha Development

Zone, a growth engine for the city in future.

7 overseas professional incubation (Science and Technology) parks All these parks are actively providing support and incubation services for the overseas entrepreneurs that establish business in Guangzhou.

— One Stop Services

Guangzhou Service Center for Scholarly Exchange (GZSCSE) is established by the city to service the overseas professionals in Guangzhou. The services provided by the center include qualification certification, diploma certification, entrepreneurship training, employment guidance, Human Resources services etc.



Karlsruher Institut für Technologie

Karlsruhe Institute of Technology (KIT) is the result of the merger of University Karlsruhe and Research Center Karlsruhe. It is a unique institution in Germany, which combines the mission of a university with that of a large-scale research center of the Helmholtz Association. With 8,000 employees and an annual budget of EUR 650 millions, KIT is one of the largest research and education institutions worldwide.

For our **Institute of Functional Interfaces (IFG)**, we wish to employ a

Scientist (f/m) in Physical Chemistry or Bioengineering

under an employment contract limited up to 3 years.

The IFG plans to establish a Laboratory for Molecular Haemocompatibility. We are looking for a Ph.D scientist with a strong background in biophysical chemistry, bioengineering and/or biomaterials, and with a solid knowledge in infrared and X-ray spectroscopy and Circular Dichroism Spectroscopy.

The successful applicant should also be familiar with the concepts in Molecular Biology and Surface Science. You have to participate in grant applications and apply for third party funding, establish and support collaborations with clinical workgroups, to supervise PhD students and post-docs, and to contribute to the teaching duties of the IFG.

We offer a challenging scientific task with a high degree of autonomy, a variety of training options, and the use of latest technical equipment.

KIT attaches great importance to equal opportunities of female and male employees. We would therefore kindly encourage female applicants to apply for this job. If qualified, handicapped applicants will be preferred.

Kindly apply via our online application system by **November 30th, 2012** or send your application documents to Mr Gehring, Human Resources, indicating the **vacancy number 463/2012** and the **ID number 8**. For technical information, please contact Prof. Dr. Grunze, phone +49 (0) 721 608-23934 (michael.grunze@urz.uni-heidelberg.de).

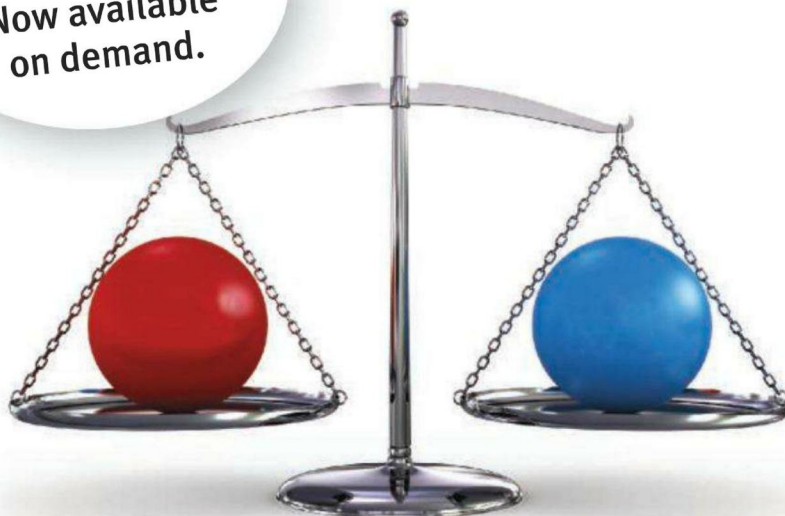
Karlsruhe Institute of Technology - Human Resources - Postfach 36 40 - 76021 Karlsruhe, Germany

Further relevant details can be found on our homepage under www.kit.edu

KIT – University of the State of Baden-Württemberg and National Laboratory of the Helmholtz Association

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Science Careers

From the journal *Science*



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**Director of Institute for Bioscience and
Biotechnology Research-IBBR
Position #118732**

University of Maryland and
National Institute of Standards and Technology

The University of Maryland and the National Institute of Standards and Technology are seeking an outstanding individual to serve as Director of the *Institute for Bioscience and Biotechnology Research (IBBR)* (www.ibbr.umd.edu) with the vision of making IBBR a premier biotechnology research institute. The research strengths of IBBR are in the areas of (1) structural biology (2) biomolecular engineering and biofabrication (3) host-pathogen interactions and (4) therapeutic design and development. A primary role of the new Director will be to lead and significantly expand a joint NIST/UM research effort that combines basic, measurement and translational research for the development, manufacturing and standardization of advanced therapeutics and supporting diagnostics.

Applicants and nominees should submit a letter of interest; resume; and the names, addresses, and telephone numbers of at least four persons who can be contacted by the search committee for reference. Confidential review of nominations and applications will begin Monday, **November 26, 2012** and will continue until the position is filled.

For application requirements, the complete position description, and to apply go online: jobs.umd.edu/applicants/Central?quickFind=58047. For more information contact: **Ms. Sandy Davis, IBBR Director Search Coordinator, 1119 Main Administration Building, University of Maryland, College Park, MD 20742-5031**, or electronically to sandyd@umd.edu.

The University of Maryland is an Equal Opportunity Employer; women and minorities are encouraged to apply.



The
UNIVERSITY
of **VERMONT**

The Department of Medical Laboratory and Radiation Sciences at the University of Vermont is seeking an outstanding individual to make a significant contribution to the medical laboratory science program in the College of Nursing and Health Sciences. This full-time, nine-month, tenure track position is open to PhDs or those with an equivalent doctoral degree focused in the medical laboratory sciences or related area. Possession of an MLS, MT or CLS credential is highly desirable. The successful candidate will have a strong commitment to undergraduate education including advising, and will be an active participant in a PhD program either within the College of Nursing and Health Sciences or on campus. The successful candidate will be positioned to generate extramural funding and implement a sustained program of active research. Candidates whose research focus aligns with the research interests of the Vermont Cancer Center (VCC) will have the potential to collaborate with VCC. A strong commitment to diversity, interdisciplinary research, and inter-professional education are essential attributes. Unique opportunities for academic leadership may be available with this position.

Prospective candidates should apply online at www.uvmjobs.com. Search for the position using the department name **Medical Lab & Radiation Sci/56030 posting #0040637**. Attach curriculum vitae and letter detailing areas of expertise and professional goals. The position will remain open until filled and review of applications will begin December 14th. All inquiries will remain confidential. For additional information please contact **Paula Deming, PhD, MT, paula.deming@uvm.edu, (802) 656-2506**.

The University of Vermont is an Affirmative Action/Equal Opportunity Employer and encourages candidates of women people of color and people with disabilities.

**Recruitment Announcement
of China National Center for Food Safety Risk
Assessment for High-level Scholars and Talents**

China National Center for Food Safety Risk Assessment (CFSA) was established on Oct. 13, 2011, located in Beijing. It is the national technical institution for food safety risk assessment, surveillance, alert, communication and food safety standards. CFSA sincerely welcomes the innovative and honest outstanding talents in food related fields to join us.

A. Job titles and requirements

a. Leading Scholar

Candidates: member of Chinese Academy of Sciences, member of Chinese Academy of Engineering, selected candidates of The Thousand Talents Program

Backgrounds: Food safety related fields as medical science, chemistry, agriculture, food science, microbiology, pharmacology and etc.

Qualifications and conditions:

- Academic attainments and influences
- With international perspectives and be innovative
- Fit to qualifications and professional needs
- Good morals and professional qualities
- Good health conditions

b. Leading Scientist

Candidates: Yangtze River Scholars, Recipients of National Science Fund for Distinguished Young Scholars, Scientists selected by New Century National Hundred, Thousand and Ten Thousand Talent Project, Provincial and ministerial level experts with outstanding contributions, Professors and associate professors from universities, colleges as well as science institutes

Backgrounds: Microbiology, epidemiological statistics, toxicology, information technology, environmental science, nutrition and food hygiene

Qualifications and conditions:

- With international perspectives and be innovative
- Advanced management concepts and organization capacity
- Extensive experience
- Good morals and professional qualities
- Good health conditions
- Applicants for full-time positions should be under 55.
- Part-time positions and "Double span" mode could be considered for those who can not maintain a full-time status with CFSA.

B. Benefits and compensations

CFSA shall provide the employee with competitive salary and housing compensation, as well as kickoff research funds and other conditions.

C. Application materials

- a. Application form (see attachment), Degree certificates, CV, Copies of certificates for major positions
- b. Preliminary work plan, including goals and budget
- c. List of scholarly articles, treatises or patents that can prove the academic achievements in recent 5 years, 3-5 copies of representative articles
- d. Listed scholarly articles should be published on Chinese core journals or science and technology core journals, or collected by SCI, EI, SSCI.
- e. Major awards and copies of certificates
- f. The materials of research projects including project titles, the meanings of research, the role of the applicant, the origin of the project and the financial conditions

D. Notes

For more information please see <http://www.cfsa.net.cn> to download the application form. For submission of application materials, please send to shpzhx303@126.com, with the subject as "name+Application".

POSITIONS OPEN



FACULTY POSITION in HIV/AIDS Research at Baylor College of Medicine

The Department of Molecular Virology and Microbiology at Baylor College of Medicine invites applications for a tenured or tenure-track faculty position focused on any area of HIV/AIDS research. The preferred candidate will study HIV pathogenesis, HIV-related malignancies, or HIV immunology. Rank will be dependent upon qualifications. Preference will be given to applicants with an established research program. For a tenured position, the candidate must have an established reputation and an outstanding funded research program. The successful candidate will be expected to maintain an innovative funded research program and to participate in graduate training. Generous startup funds are available. This is an opportunity to join an institution with a unique collaborative spirit and a dedication to excellence in research, education, and patient care. Baylor College of Medicine is located in the rich scientific setting of the renowned Texas Medical Center, the largest incorporated medical center in the world, in Houston, a dynamic and ethnically diverse city with many cultural amenities. Current research interests of departmental faculty focus on viral and bacterial gene expression, microbial pathogenesis, RNA and DNA viruses of human diseases, viral oncology, vaccines, the microbiome, and host-microbe interactions. State-of-the-art core facilities are available. Multidisciplinary research centers, including the Center for AIDS Research, the Dan L. Duncan Cancer Center, the Alkek Center for Metagenomics and Microbiome Research, the Vaccine Research Center, and the Digestive Diseases Center, offer excellent opportunities for collaborations. Applicants should submit curriculum vitae, a statement of research accomplishments and future plans, a description of teaching experience and philosophy, and names and addresses of three references by January 31, 2013 to: **Search Committee, Department of Molecular Virology and Microbiology, Mail Stop: BCM385, Baylor College of Medicine, Houston, TX 77030.** Electronic submission preferred at **e-mail: bcm-mvm-facultypos6@bcm.edu.** *Baylor College of Medicine is an Equal Opportunity/Affirmative Action/Equal Access Employer.*

TENURE-TRACK FACULTY POSITION Biomedical Sciences Department of Biological Sciences The University of Southern Mississippi

The Department of Biological Sciences at The University of Southern Mississippi (**website: www.usm.edu/biology/**) invites applications for a tenure-track **ASSISTANT PROFESSOR** position in basic biomedical sciences. The successful candidates will join our growing and collaborative microbiology/molecular biology group in the Department of Biological Sciences and across the College of Science and Technology. We seek outstanding candidates who are applying innovative molecular, cellular and/or 'omics-related approaches within the area of biomedical sciences—broadly defined in such areas as microbiology, physiology, cancer biology and biomedical engineering. Successful candidates are expected to develop an independent, extramurally funded research program, mentor graduate students, and participate in undergraduate/graduate teaching in their areas of expertise. Applicants must hold an earned Ph.D. in an appropriate field and have relevant postdoctoral experience. A competitive salary commensurate with qualifications and experience, competitive startup package, laboratory space, and state-of-the-art facilities will be provided. The position will begin August 2013. Applications must be submitted online at **website: <https://jobs.usm.edu>** (job posting #0002471). Review of applications will begin January 21, 2013, and will continue until the position is filled. For inquiries about the position, contact **Dr. Mohamed Elasri**, Search Committee chair, at **telephone: 601-266-6916** or **e-mail: mohamed.elasri@usm.edu**.

Affirmative Action/Equal Opportunity Employer/ADA.

POSITIONS OPEN



FACULTY POSITIONS University of California San Diego Department of Bioengineering

The Department of Bioengineering in the Jacobs School of Engineering at the University of California, San Diego is inviting applications for one or more tenure-track or tenured faculty positions at the **ASSISTANT PROFESSOR, ASSOCIATE PROFESSOR, or FULL PROFESSOR** levels. To obtain more information or submit your application materials, go to **website: <https://apol-recruit.ucsd.edu/apply>** and apply to **position number 10-507.**

ASSISTANT PROFESSOR in Biochemistry

Georgetown University wishes to recruit a tenure-track Assistant Professor in Biochemistry to begin fall 2013, who will be housed in Regents Hall, a newly opened, state-of-the-art science center. Research areas in biochemistry as broadly defined will be considered that complement those of existing faculty in the Department. Research interests related to sustainability and energy or xenobiotics and the environment are especially welcome. Candidates must have a Ph.D. degree in chemistry or a closely related field and postdoctoral training or equivalent is desirable. Development of an internationally recognized, externally funded research program and teaching at the undergraduate and graduate levels, particularly biochemistry sequences, are expected. Please send curriculum vitae, description of research plans, statement of teaching philosophy as one document in PDF format, and arrange for three letters of recommendation to be sent to **e-mail: chemsearch@georgetown.edu**. For full consideration, complete applications should arrive before December 15, 2012. *Georgetown University is an Equal Opportunity/Affirmative Action Employer fully dedicated to achieving a diverse faculty and staff; applications from qualified women and minority candidates are encouraged.*

TENURE-TRACK FACULTY POSITION Microbiology Department of Biological Sciences The University of Southern Mississippi

The Department of Biological Sciences at The University of Southern Mississippi (**website: <http://www.usm.edu/biology/>**) invites applications for a tenure-track **ASSISTANT PROFESSOR** position in microbiology. The successful candidate will join our growing and collaborative microbiology/molecular biology group in the Department of Biological Sciences and across the College of Science and Technology. We seek outstanding candidates who are engaged in innovative microbiology research. Successful candidates are expected to develop an independent, extramurally funded research program, mentor graduate students, and participate in undergraduate/graduate teaching in their areas of expertise. Applicants must hold an earned Ph.D. in an appropriate field and have relevant postdoctoral experience. A competitive salary commensurate with qualifications and experience, competitive startup package, laboratory space, and state-of-the-art facilities will be provided. The position will begin August 2013. Applications must be submitted online at **website: <https://jobs.usm.edu>** (job posting #0002472). Review of applications will begin January 21, 2013, and will continue until the position is filled. For inquiries about the position, contact **Dr. Tim McLean**, Search Committee chair, at **telephone: 601-266-4753** or **e-mail: timothy.mclean@usm.edu**.

Affirmative Action/Equal Opportunity Employer/ADA.

POSITIONS OPEN



The Center for Cardiovascular Sciences invites applications for tenure-track **ASSISTANT or ASSOCIATE PROFESSORS**. We seek highly motivated individuals with translatable research programs in areas including regenerative medicine, tissue engineering, and genomics. The applicant's research should complement existing programs in the Center that include vascular smooth muscle and endothelial cell signaling and function, vascular remodeling, airway hyper-reactivity, and cardiac development. Applicants should have interests in graduate and postgraduate mentoring and medical teaching. An M.D., Ph.D., or combined degree and three years of productive postdoctoral experience are required for appointment at the Assistant Professor level. Applicants for Associate Professor should have three to five years of experience as an Assistant Professor and a nationally recognized research program. Physician scientists will be jointly recruited with an appropriate clinical department.

Opportunities for research collaboration include participation in the Capital Region Research Alliance involving scientists at AMC, Rensselaer Polytechnic Institute, and UAlbany. The area offers diverse cultural and recreational attractions with easy access to Boston, New York City, and the Adirondack, Catskill, and Berkshire Mountains. For further information about the Center and Albany Medical College, please visit **website: <http://www.amc.edu>**.

Applicants should submit current curriculum vitae, description of research interests and plans, and names of three references in PDF format by January 18, 2013 to **e-mail: viennew@mail.amc.edu**.

An Equal Opportunity/Affirmative Action Employer. Women and Minorities are encouraged to apply.



SMITHSONIAN INSTITUTION FELLOWSHIP PROGRAM

GRADUATE STUDENT, PREDOCTORAL, POSTDOCTORAL, and SENIOR FELLOWSHIPS in Animal behavior, ecology, and environmental science; including an emphasis on the tropics; Earth sciences and paleobiology; Evolutionary and systematic biology; History of science and technology. Tenable in residence at the Smithsonian facilities. Stipends and tenure vary. Awards are contingent upon the availability of funds. Deadline: January 15 annually. Contact: Office of Fellowships and Internships, Smithsonian Institution, Washington, DC, **telephone: 202-633-7070**, **e-mail: siofg@si.edu**, **website: <http://www.si.edu/ofi>**. *An Equal Opportunity Employer.*



JAMES SMITHSON FELLOWSHIP PROGRAM

The James Smithson Fellowship provides postdoctoral scholars in the fields of science, the humanities, and the arts an interface between research/scholarship and policy. Tailored to applicants' interests, Smithsonian fellowships combine the best of the Smithsonian's vast scholarship and collections with unparalleled access to the nation's capital in a yearlong experience. Deadline: January 15, 2013. Contact: Office of Fellowships and Internships, Smithsonian Institution Washington, DC **telephone: 202-633-7070**, **e-mail: siofg@si.edu**, **website: <http://www.si.edu/ofi/jamessmithson>**. *An Equal Opportunity Employer.*



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To learn more, visit aaas.org/plusyou/fellows



POSITIONS OPEN

PHYSICIAN-SCIENTIST INVESTIGATOR in Endocrinology Weill Cornell Medical College

The Division of Endocrinology of the Department of Medicine, Weill Cornell Medical College, has a tenure-track faculty position available for a junior investigator working in the general area of CNS regulation of metabolism. Candidates are expected to qualify for a faculty position at the **ASSISTANT PROFESSOR** level at Weill Cornell Medical College, and to have or will have NIH funding for their research. Physician-scientists with M.D. or combined M.D.-Ph.D. degrees are preferred. Competitive startup packages and laboratory resources will be provided. Applications will be considered until December 31, 2012.

Interested candidates should send their curriculum vitae to **David J. Christini**, Ph.D., Chair of Endocrinology Research Search Committee, c/o **Beverly Borg**, e-mail: bborg@med.cornell.edu or by mail to: **525 East 68th Street, Box 130, Room M-522, New York, NY 10065**.

Equal Opportunity Employer/Minorities/Females/Persons with Disabilities/Veterans.

University of Hawaii at Manoa Cancer Center: **ASSISTANT/ASSOCIATE/FULL RESEARCHER** (Epidemiology Program), **Position #86188**, Tenure-track, Closes November 21, 2012. Refer to **website: <http://www.pers.hawaii.edu/wuh/>** for complete information. *The University of Hawaii is an Equal Opportunity/Affirmative Action Institution. Women and minorities are encouraged to apply.*

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From technology specialists to patent attorneys to policy advisers, learn more about the types of careers that scientists can pursue and the skills needed in order to succeed in nonresearch careers.

Science Careers

From the journal *Science* **AAAS**

Announcing our new partnership with NASA Federal Credit Union

Dear Member:

AAAS is committed to offering you member benefits that fit your needs and make your membership more valuable.

With that in mind AAAS is embarking on a new partnership with NASA Federal Credit Union that will provide you with access to a wide range of financial tools and products. Like AAAS, NASA Federal Credit Union is dedicated to serving the scientific community. This shared perspective is just one of the many reasons that we are embarking on this partnership.

As you may know, we have recently ended our banking relationship with Bank of America, but we're confident that our new partnership with NASA FCU will provide you with a superior banking experience. NASA FCU offers members better ways to save and smarter ways to borrow with friendly, professional service – along with anytime, anywhere account access.

Moreover, unlike other financial institutions that have public stockholders, NASA FCU is a not-for-profit financial cooperative where being a member means being an owner, too. And as a member/owner, you will enjoy unique benefits like: better loan rates, higher dividends and state-of-the-art products and services.

We'll be sending you more information about this great new benefit over the coming months. In the meantime, be sure to visit nasafcu.com/AAASpackage to apply for the new AAAS Platinum Advantage Rewards or Platinum Cash Rewards credit cards. You can also take a sneak peek at the AAAS Check Card and Checks coming soon.

Sincerely,

A handwritten signature in blue ink, appearing to read 'Ian King'.

Ian King
Director of Marketing and Membership, AAAS

